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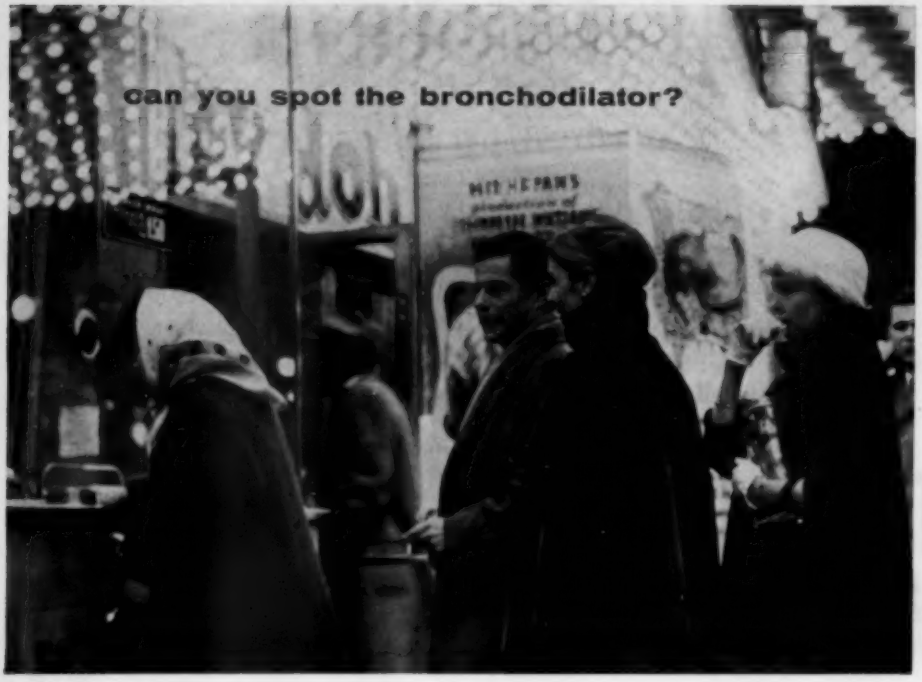

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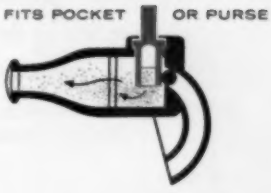

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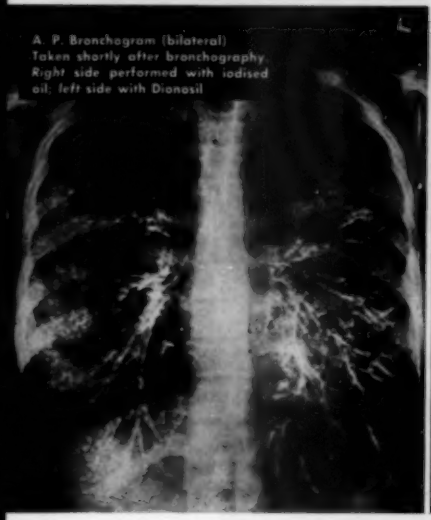

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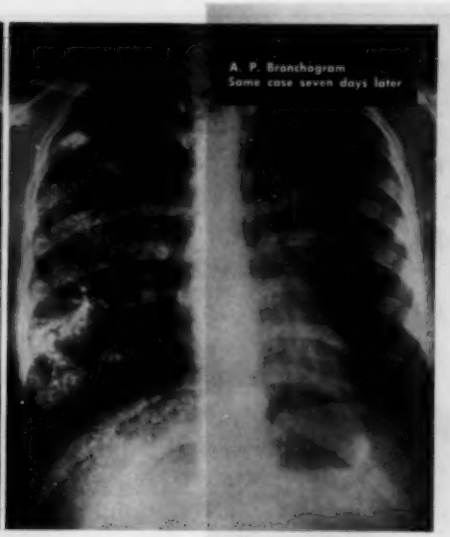

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R. A. Green and D. J. Coleman: "Cuirass Respirator for Endoscopy." ANESTHESIA **10**:369-373, October, 1955.

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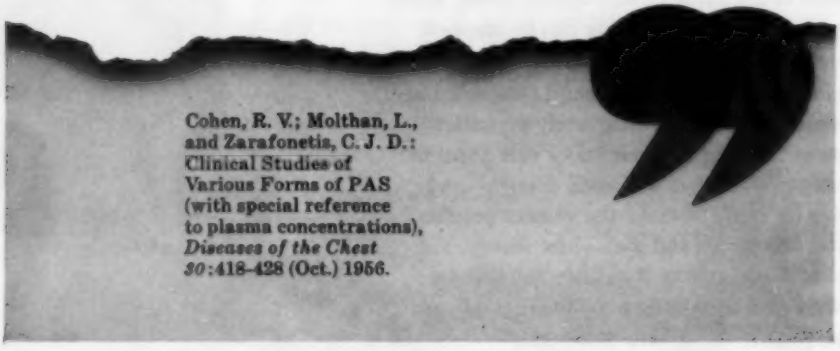

Cohen, R. V.; Molthan, L., and Zarafonetis, C. J. D.: Clinical Studies of Various Forms of PAS (with special reference to plasma concentrations), *Diseases of the Chest* *30*:418-428 (Oct.) 1956.

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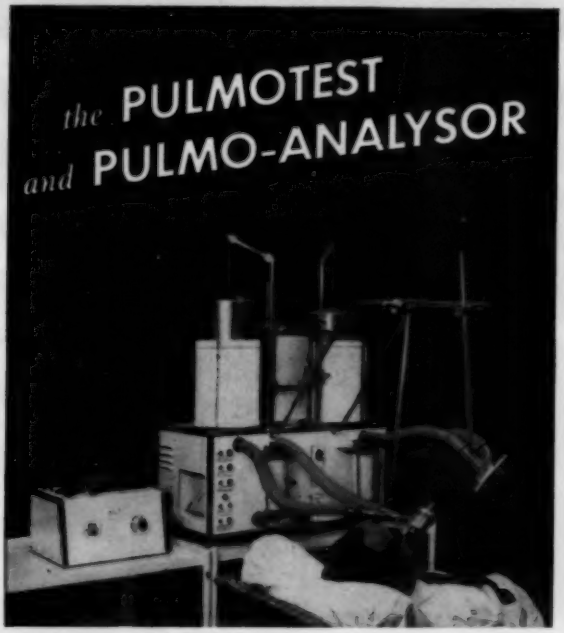

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ERRATUM

In Volume 75, No. 1, 1957, in the article entitled "The Drug Treatment of Nonhospitalized Patients with Tuberculosis" by Arthur D. Robins *et al.*, the final sentence of the paragraph preceding the Discussion on page 47 should read as follows:

> "In addition, tubercle bacilli could no longer be recovered at the end of two years from the sputum *of 12 of 94* patients who were still discharging bacilli after one year of chemotherapy, a 'late conversion rate' of almost 13 per cent (table 10, line 2)."

THE AMERICAN REVIEW OF TUBERCULOSIS AND PULMONARY DISEASES

VOLUME 75 April, 1957 NUMBER 4

THE METABOLISM OF NICOTINIC ACID IN MYCOBACTERIA[1]

A Method for Differentiating Tubercle Bacilli of Human Origin from Other Mycobacteria

KIYOSHI KONNO, RUDOLF KURZMANN, AND KENNETH T. BIRD

(Received for publication July 2, 1956)

INTRODUCTION

It is well known that nicotinic acid and its compounds play an important role in bacterial metabolism. Coenzyme I and II (di- and tri-phosphopyridine nucleotide) are derived from nicotinic acid and are the most important "hydrogen carriers" within the living cell. Thus, nicotinic acid is an essential growth factor for many bacteria. Certain bacteria, e.g., *Staphylococcus aureus*, *Corynebacterium diphtheriae*, and *Bacillus dysenteriae*, require the addition of nicotinic acid to synthetic culture media because of their inability to synthesize nicotinic acid. Other organisms, such as *Hemophilus influenzae*, require the addition of coenzyme to the culture medium for growth because they are unable to synthesize coenzyme from either nicotinic acid or nicotinic acid amide. Tubercle bacilli, however, have the ability to synthesize nicotinic acid from certain amino acids, e.g., asparagine and glutamic acid, if they are the only source of nitrogen in the culture medium; neither coenzyme nor nicotinic acid need be added to the culture medium for growth.

Pope and Smith (1) determined several constituents of the vitamin B complex family (thiamine, riboflavin, biotin, and nicotinic acid) as encountered in the culture filtrate of a human and a bovine strain of tubercle bacilli and found that the human strain (H37Rv) produced some of these substances in appreciably larger amounts than did the bovine strain (Ravenel). The difference was most marked for nicotinic acid. Bird (2) found higher values of nicotinic acid in the hydrolyzed whole culture than in the nonhydrolyzed culture filtrate of human tubercle bacilli H37Rv. One of the present writers (3) compared the production of nicotinic acid by various types of mycobacteria and confirmed that the culture filtrates of human tubercle bacilli are considerably richer in nicotinic acid than the filtrates of other types of mycobacteria.

It is the purpose of this paper to report further observations on a number of human, bovine, and avian tubercle bacilli and on nonpathogenic mycobacteria

[1] From the Cambridge Sanatorium, Cambridge, Massachusetts; and the Research Institute for Tuberculosis and Leprosy, Tohoku University, Sendai, Japan.

obtained from several laboratories in the United States. Results will also be described relative to the question whether the amount of nicotinic acid synthesized differs in virulent and attenuated tubercle bacilli of human and bovine origin and in isoniazid-susceptible and isoniazid-resistant strains.

Materials and Methods

A total of 32 strains of mycobacteria from various sources was studied. The standard laboratory strains used were:

Tubercle bacilli of human origin:
Pathogenic strains:
H37Rv (from Trudeau Laboratory, Trudeau, New York)
Campbell (from Trudeau Laboratory, Trudeau, New York)
Erdman (from Trudeau Laboratory, Trudeau, New York)
Pearson Type I (from National Jewish Hospital, Denver, Colorado)
Attenuated strains:
H37Ra (from Trudeau Laboratory, Trudeau, New York)
JH16Ra (from Trudeau Laboratory, Trudeau, New York)
JH6Ra (from Trudeau Laboratory, Trudeau, New York)
Tubercle bacilli of bovine origin:
Pathogenic strains:
Vallée (from National Jewish Hospital, Denver, Colorado)
Ravenel (from Trudeau Laboratory, Trudeau, New York)
Attenuated strain:
BCG (from Henry Phipps Institute, Philadelphia, Pennsylvania)
Tubercle bacilli of avian origin:
Sheard (from Trudeau Laboratory, Trudeau, New York)
Camden (from Public Health Research Institute, New York)
Avian I (from National Jewish Hospital, Denver, Colorado)
Nonpathogenic acid-fast bacilli:
M. ranae (from Trudeau Laboratory, Trudeau, New York)
M. phlei (from Trudeau Laboratory, Trudeau, New York)
M. 607 (from Trudeau Laboratory, Trudeau, New York)

The strains isolated from patients were:

a) Thirteen strains of isoniazid-susceptible tubercle bacilli obtained from cases of active pulmonary tuberculosis:

3243	3169	3199
3175	3243	3121
3173	S. A.	K. K.
G. P.	K. C.	M. A.
J. M.		

b) Three strains of isoniazid-resistant catalase-deficient tubercle bacilli

J. G.	J. F.	J. K.

Culture medium: A modified Sauton synthetic culture medium was used. This contained KH_2PO_4, 1.5 gm.; $MgSO_4 \cdot 7H_2O$, 0.5 gm.; citric acid, 1.0 gm.; asparagine, 5.0 gm.; glycerol, 50.0 ml.; and distilled water, 950.0 ml. The pH was adjusted to 7.2 with concentrated ammonia solution. Each 50 ml. of medium was divided into 100 ml. Erlenmeyer flasks and autoclaved at 20 pounds of pressure for fifteen minutes.

Periods of culture: All strains were inoculated on the surface of this medium and were incubated for forty days except in the case of nonpathogenic acid-fast bacilli, which were incubated for a period of only ten days. After incubation, the culture medium was sterilized at 100°C. for one hour and filtered through filter paper. The bacilli were then washed with a small amount of distilled water and the volume was adjusted to about 45 ml.

Methods for Determination of Nicotinic Acid

A modified Muller and Fox (4) method was used for the determination of nicotinic acid. Two milliliters of 40 per cent NaOH were added to the culture filtrate and the filtrate was brought to 50 ml. by the addition of distilled water. The solution was heated in a boiling water bath for one hour. This procedure hydrolyzes the coenzyme, nicotinamide, and nicotinic acid precursor in the culture filtrate to nicotinic acid. Then 2 milliliters of buffered ammonia solution[2] were added to 1 ml. of hydrolyzed culture filtrate and the solution was mixed. Two milliliters of 10 per cent cyanogen bromide[3] were added; the sample tube was well shaken and placed in a colorimeter within one and one-half minutes. Readings were made of the maximal yellow color intensity usually obtained in two to two and one-half minutes, using a 430 mμ filter. A Bausch and Lomb electrophotometer was used in this experiment. As a blank control, 1 ml. of hydrolyzed culture filtrate with 2 ml. of buffered ammonia were used. Two milliliters of distilled water were substituted for the 2 ml. of 10 per cent cyanogen bromide. A standard curve was established with known amounts of nicotinic acid in modified Sauton medium after the addition of 10 per cent cyanogen bromide and ammonia buffer.

Fourteen strains of tubercle bacilli as listed below were used for determination of nicotinic acid content within the cell:

H37Rv	Erdman	H37Ra	JH16Ra	Ravenel
Vallée	BCG	Sheard	*M. phlei*	*M. 607*
M. ranae	J. G.	3240	M. A.	

Fifty milligrams of dried bacilli were hydrolyzed by adding 50 ml. of 0.5 N.NaOH and autoclaving at 20 pounds of pressure for twenty minutes. The hydrolyzed solution was filtered and 1 ml. of filtrate was examined by the method outlined above for nicotinic acid.

Results

Tubercle bacilli of human origin (laboratory strains) (table 1, figures 1 and 2): Of the 7 strains of tubercle bacilli of human origin that were studied, nicotinic acid production ranged from 13.5 γ to 32.0 γ per ml. of the culture filtrate or 6.3 γ to 12.4 γ per mg. of dried bacteria.

No difference in nicotinic acid production was encountered between virulent and attenuated human tubercle bacilli.

Tubercle bacilli of bovine origin (table 1, figures 1 and 2): In contrast to the findings in human tubercle bacilli, only a minimal amount of nicotinic acid was detected in the culture media of bovine tubercle bacilli. The Vallée, Ravenel, and BCG strains showed only 1.0 γ, 1.5 γ, and 1.0 γ of nicotinic acid per ml. of

[2] Buffered ammonia solution: To a 1-liter volumetric flask containing about 500 ml. of distilled water add 87.0 gm. of K_2HPO_4, 107.0 gm. of NH_4Cl, and 6.7 ml. of concentrated ammonia and make the volume with water. Filter if sediment forms upon standing.

[3] Cyanogen bromide 10 per cent: Dissolve 50 gm. of cyanogen bromide in 500 ml. of distilled water. Both reagents remain stable for at least several months if kept in the refrigerator.

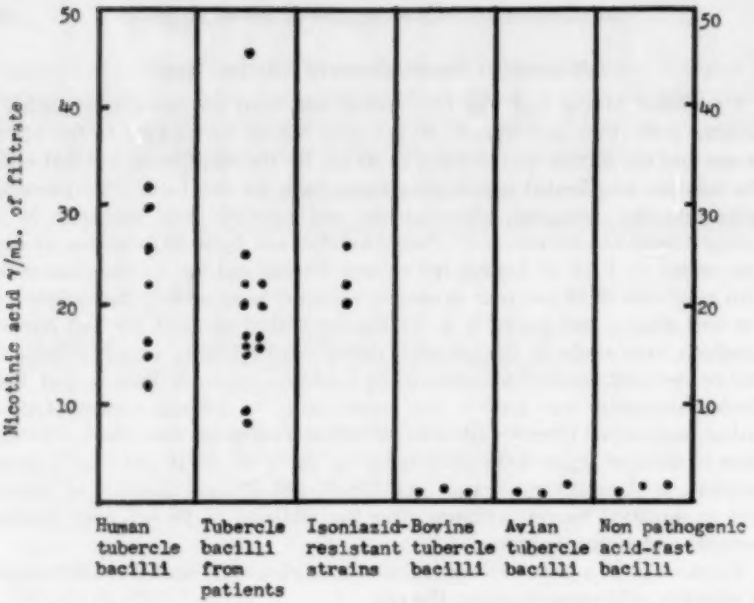

FIG. 1. Nicotinic acid synthesis of mycobacteria on modified Sauton synthetic culture media (γ per ml. of filtrate).

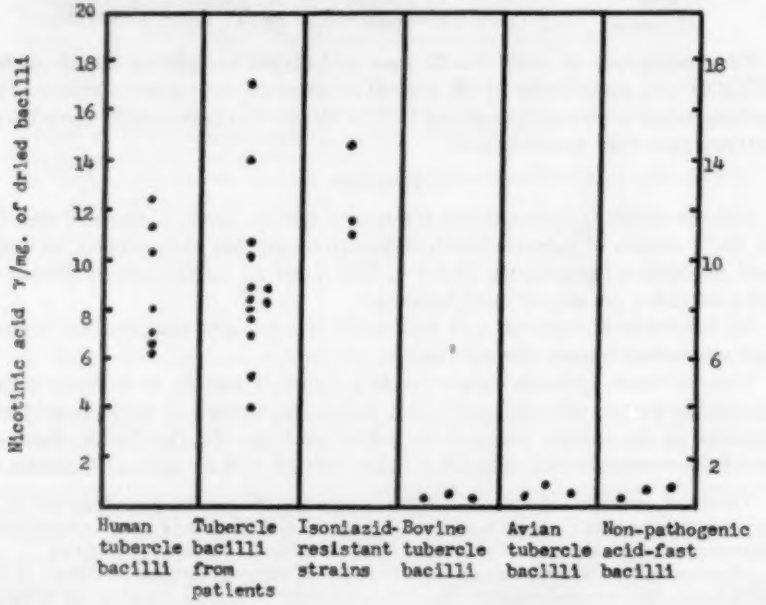

FIG. 2. Nicotinic acid synthesis of mycobacteria on modified Sauton synthetic culture media (γ per mg. of dried bacilli).

TABLE 1

NICOTINIC ACID PRODUCTION OF MYCOBACTERIA

Mycobacteria	Strain	Dried Bacilli	Nicotinic Acid γ/Ml. of Culture Filtrate	Nicotinic Acid γ/Mg. of Dried Bacilli
		mg.		
Human tubercle bacilli (40-day culture)	Virulent			
	H37Rv	120	30.0	12.4
	Campbell	100	13.5	6.7
	Erdman	108	22.0	10.2
	Pearson I	116	16.0	6.9
	Attenuated			
	H37Ra	115	26.0	11.3
	JH16Ra	190	32.0	8.4
	JH6Ra	118	15.0	6.3
Bovine tubercle bacilli (40-day culture)	Virulent			
	Vallée	150	1.0	0.3
	Ravenel	140	1.5	0.5
	Attenuated			
	BCG	136	1.0	0.4
Avian tubercle bacilli (40-day culture)	Sheard	130	1.0	0.4
	Camden	104	2.0	0.9
	Avian I	80	1.0	0.6
Nonpathogenic mycobacteria (10-day culture)	*M. ranae*	160	1.0	0.3
	M. phlei	125	1.8	0.7
	M. 607	135	2.0	0.8

culture filtrate each, or 0.3 γ, 0.5 γ, and 0.4 γ per mg. of dried bacilli. No difference in production of nicotinic acid was observed between virulent and attenuated strains.

Tubercle bacilli of avian origin (table 1, figures 1 and 2): Avian tubercle bacilli also produced only very small amounts of nicotinic acid. The Sheard, Camden, and Avian I strains produced 1.0 γ, 2.0 γ, and 1.0 γ of nicotinic acid per ml. of culture filtrate, or 0.4 γ, 0.9 γ, and 0.6 γ per mg. of dried bacilli.

Nonpathogenic acid-fast bacilli (table 1, figures 1 and 2): Three strains of nonpathogenic acid-fast bacilli were investigated. They also showed minimal production of nicotinic acid. The *M. ranae*, *M. phlei*, and *M. 607* strains produced 1.0 γ, 1.8 γ, and 2.0 γ of nicotinic acid per ml. of culture filtrate, or 0.3 γ, 0.7 γ, and 0.8 γ per mg. of dried bacilli.

Tubercle bacilli obtained from patients (table 2, figures 1 and 2): Thirteen strains of tubercle bacilli obtained from patients were examined and all of them produced a considerable amount of nicotinic acid ranging from 8.0 γ to 45.0 γ per ml. of culture filtrate, or 4.0 γ to 17.0 γ per mg. of dried bacilli.

Isoniazid-resistant catalase-deficient tubercle bacilli (table 2, figures 1 and 2): Three strains of isoniazid-resistant catalase-deficient tubercle bacilli were studied. The bacilli obtained from patient *J. G.* caused progressive tuberculosis

TABLE 2
NICOTINIC ACID PRODUCTION OF MYCOBACTERIA

Mycobacteria	Strain	Dried Bacilli	Nicotinic Acid γ/Ml. of Culture Filtrate	Nicotinic Acid γ/Mg. of Dried Bacilli
		mg.		
Tubercle bacilli from patients (40-day culture)	3243	90	25.0	14.0
	3169	103	16.5	8.0
	3199	80	17.0	10.6
	3175	105	16.0	7.6
	3243	70	16.5	11.8
	3121	100	8.0	4.0
	3173	124	22.0	8.8
	S. A.	120	20.0	8.3
	K. K.	106	15.0	7.0
	P. G.	120	20.0	8.3
	K. C.	126	22.0	8.8
	M. A.	132	45.0	17.0
	J. M.	83	8.5	5.1
Isoniazid-resistant, catalase-deficient tubercle bacilli (40-day culture)	J. G.	96	22.0	11.4
	J. F.	88	26.0	14.7
	J. K.	90	20.0	11.1

TABLE 3
NICOTINIC ACID IN THE DRIED BACILLI

(Hydrolyzed with 0.5N-NaOH; Autoclaved at 20 Pounds for Twenty Minutes)

Strains	Nicotinic Acid	Strains	Nicotinic Acid
	γ/mg.		γ/mg.
H37Rv	0.2	Sheard	0
Erdman	0	*M. phlei*	0.1
H37Ra	0.3	*M. 607*	0
JH16Ra	0	*M. ranae*	0
Ravenel	0	J. G.	0.2
Vallée	0	3240	0
BCG	0	M. A.	0

in the guinea pig (lymph nodes, lung, liver, spleen). The other two strains were nonpathogenic for guinea pigs. The nicotinic acid production of these strains was identical with that of the human strains and ranged from 20.0 γ to 26.0 γ per ml. of the culture filtrate, or from 11.1 γ to 14.7 γ per mg. of dried bacilli.

Nicotinic acid in the dried bacilli (table 3): All of the 14 strains of hydrolyzed acid-fast bacilli showed either no, or minimal, traces of nicotinic acid in the bacilli, e.g., 0 to 0.3 γ per 1 mg. of dried bacilli.

DISCUSSION

It is apparent that there is a marked quantitative difference in the ability of various bacteria to synthesize nicotinic acid from the nitrogen sources of their culture media. The results of the present investigation suggest that among the

mycobacteria only tubercle bacilli of human origin can produce a significant amount of nicotinic acid from the amino acids of the culture media. If, in the chain of metabolic processes, nicotinic acid is the precursor of coenzyme, the tubercle bacilli of human origin might have an oxidation enzyme system quite different from that of other mycobacteria. Most interesting and significant is the difference in nicotinic acid production between human and bovine tubercle bacilli. Probably of more important significance is the difference between human tubercle bacilli and the nonpathogenic mycobacteria.

Hitherto, two methods of differentiation of human from bovine tubercle bacilli have been available: the behavior on culture media and the inoculation of suitable animals. Bovine tubercle bacilli grow more slowly and do not require the addition of glycerol to the culture medium. Moreover, bovine bacilli are generally more pathogenic for the rabbit than are the human bacilli.

The method described in the present report is an additional simple and accurate way to differentiate human tubercle bacilli and other mycobacteria, including bovine tubercle bacilli.

The color difference with the method used is more clearly apparent than would be expected from the comparison of the mere figures. The culture filtrates of human bacilli develop a yellow color, whereas other mycobacteria develop either no color or a barely visible light yellow color. This striking difference may allow this method to be applied by the clinician without colorimetric quantitation.

The ability of nicotinic acid production bears no apparent relationship to the virulence of the bacillus since there was no difference in the production of nicotinic acid in the virulent or in the attenuated strains. This applies to both the human tubercle bacilli and the bovine. It is of interest that the BCG strain which is highly attenuated and which has lost its original cultural and pathogenic properties still has the characteristics of virulent bovine strains so far as the nicotinic acid synthesis is concerned.

It is of special interest that isoniazid-resistant bacilli studied fail to show any difference in the nicotinic acid production regardless of their catalase content or their pathogenicity for experimental animals.

In short, it appears that the faculty of synthesizing nicotinic acid is linked to the original animal organism and not to the pathogenicity of the bacilli.

There were only minimal traces of nicotinic acid detectable in the hydrolyzed bacilli of 14 strains. Hence, it is assumed that, during the processes of sterilization, filtration, and washing, the nicotinic acid passes almost completely from the bacilli into the filtrate.

Summary

Seven laboratory strains of tubercle bacilli of human origin, 3 strains of tubercle bacilli of bovine origin, 3 strains of tubercle bacilli of avian origin, 3 strains of nonpathogenic acid-fast bacilli, 13 strains of tubercle bacilli from tuberculous patients, and 3 strains of isoniazid-resistant bacilli were studied with respect to nicotinic acid synthesis in modified Sauton liquid culture medium.

There was a striking difference in the nicotinic acid synthesis between human and other types of mycobacterium. Human tubercle bacilli produced more than 13.5 γ per ml. of culture filtrate, or 6.3 γ per mg. of dry bacillary weight, while the other mycobacteria, including bovine tubercle bacilli, did not exceed 2.0 γ per ml., or 1.0 γ per mg. of dry bacillary weight. Among the two forms of tubercle bacilli which are pathogenic for the human, the bovine is far less capable or producing nicotinic acid.

There was no difference in the nicotinic acid production between virulent and attenuated bacilli of human or bovine origin.

All strains of tubercle bacilli obtained from tuberculous patients were comparable in their ability to synthesize nicotinic acid. Thus there was no difference in the nicotinic acid production of the isoniazid-susceptible and the isoniazid-resistant strains studied regardless of their catalase content or pathogenicity for the guinea pig.

Only minimal traces of nicotinic acid (0–0.3 γ per mg.) were found in the hydrolyzed bacilli.

Nicotinic acid synthesis is apparently highly specific and may serve as a method of differentiating human tubercle bacilli from other types of mycobacterium.

Sumario

El Metabolismo del Acido Nicotínico en las Micobacterias. Método para Diferenciar los Bacilos Tuberculosos Humanos de Otros Tipos de Micobacterias

Este estudio con respecto a la síntesis del ácido nicotínico en el medio de cultivo líquido de Sauton modificado comprendió 7 cepas de bacilos tuberculosos humanos del laboratorio, 3 cepas de bacilos tuberculosos bovinos, 3 cepas de bacilos tuberculosos aviarios, 13 cepas de bacilos tuberculosos procedentes de enfermos tuberculosos y 3 cepas de bacilos isoniacidorresistentes.

En la síntesis del ácido nicotínico, la diferencia es notable entre las micobacterias humanas y otras formas. Los bacilos tuberculosos humanos produjeron más de 13.5 γ por ml. de filtrado de cultivo u 6.3 γ por mg. de peso bacilar seco, en tanto que otros tipos no excedieron de 2.0 γ por ml. o 1.0 γ por mg. de peso bacilar seco. Entre los dos tipos que son patógenos para el hombre, el bovino es mucho menos capaz de producir ácido nicotínico.

No existe diferencia en la producción de ácido nicotínico entre los bacilos virulentos y los atenuados de origen humano o bovino.

Todas las cepas de bacilos obtenidas de enfermos tuberculosos resultaron comparables en su capacidad para sintetizar ácido nicotínico. No se descubrió diferencia alguna en la producción de este ácido entre las cepas susceptibles y las resistentes a la isoniacida estudiadas, independientemente de su contenido de catalasa o su patogenicidad para el cobayo.

No se encontraron más que mínimos indicios de ácido nicotínico (0–0.3 γ por mg.) en los bacilos hidrolizados, La síntesis del ácido nicotínico es aparentemente específica para cada tipo y puede servir como método para diferenciar los bacilos tuberculosos humanos de otros tipos de micobacterias.

Resume

Le métabolisme de l'acide nicotinique dans les mycobactéries. Méthode permettant de différencier les bacilles tuberculeux humains des autres types de mycobactéries

Sept souches de laboratoire de bacilles tuberculeux humains, 3 souches de bacilles tuberculeux bovins, 3 souches de bacilles tuberculeux aviaires, 3 souches de bacilles acido-resis-

tants non pathogènes, 13 souches de bacilles tuberculeux provenant de malades et 3 souches de bacilles isoniazido-résistants furent étudiées à l'égard de la synthèse de l'acide nicotinique par culture en milieu liquide du type Sauton modifié.

Il y a une différence saisissante dans la synthèse de l'acide nicotinique entre le bacille humain et les autres types de mycobactéries, les bacilles tuberculeux humains produisant plus de 13,5 γ par ml de filtrat de culture ou 6,3 γ par mg de poids bacillaire sec tandis que les autres types ne produisent pas plus de 2 γ par ml ou 1 γ par mg de poids bacillaire sec. Parmi les deux types pathogènes pour l'homme, le bovin est moins apte à produire de l'acide nicotinique.

Il n'y a aucune différence dans la production d'acide nicotinique entre les bacilles virulents ou atténués d'origine humaine ou bovine.

Toutes les souches de bacilles tuberculeux obtenues chez des malades étaient comparables quant à leur aptitude à effectuer la synthèse de l'acide nicotinique. Il n'a été démontré aucune différence dans la production d'acide nicotinique entre les souches isoniazido-sensibles ou résistantes étudiées, quelle qu'ait été leur teneur en catalase ou leur pathogénicité pour le cobaye. Il n'a été découvert que des traces minimes d'acide nicotinique (0 à 0.3 par mg) dans les bacilles hydrolyzés. La synthèse de l'acide nicotinique paraît être spécifique et peut servir de méthode de différenciation entre le bacille tuberculeux humain et les autres types de bactéries.

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A COMPARISON OF THE RESULTS OF THE SURGICAL AND THE NONSURGICAL TREATMENT OF TUBERCULOUS PATIENTS WITH THE "OPEN-NEGATIVE" SYNDROME[1,2]

JOHN W. BELL, ALFRED M. DECKER, JR., AND JAMES W. RALEIGH

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Introduction

Although chemotherapy may succeed in rendering the tuberculous patient noninfectious, antituberculous drugs commonly fail to achieve bacteriologic cure. The long-term prognosis of patients in whom annular shadows persist during treatment, in spite of early sputum "conversion," is particularly uncertain

Because the prolonged use of antimicrobial agents has resulted in an increasing number of patients with open lesions and sputum negative for tubercle bacilli, the present study was undertaken to determine the proper hospital management of such cases.

The incidence of patients displaying the "open-negative" syndrome in this hospital, observed six to eight months after the initiation of chemotherapy, is approximately 40 per cent (table 1).

These are patients who have begun treatment because of the finding of both a cavitary lesion and tubercle bacilli in the sputum. The incidence of the "open-negative" syndrome is somewhat less (33 per cent) among patients receiving antituberculous therapy for a second or third time; yet this group comprises half of the resected series in this hospital.

A similar incidence of patients achieving the "open-negative" state, during or subsequent to initial or re-treatment chemotherapy, is apparent in the data reported from individual hospitals at regional and national conferences (1, 2). Whether these patients should be treated with pulmonary resection, further chemotherapy, or merely by careful observation remains unclear from current information.

The present report is an analysis of the incidence and details of relapse in both the resected and nonsurgical "open-negative" patients, treated during the period January, 1951, to January, 1955, and a comparison of the present status of the entire group.

Material and Methods

Medical series: Of 536 consecutively admitted patients who started their initial chemotherapy prior to December 31, 1954, and completed such therapy during eight months or more without interruption, 195 (36 per cent) had persistent cavitation without tubercle bacilli in the sputum when evaluated after six to eight months of therapy. Each patient had roentgenographic evidence of cavity at the beginning of treatment and had the diagnosis of tuberculosis confirmed by accepted bacteriologic methods.

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[1] From the Veterans Administration Hospital, Sunmount, New York.

[2] Presented, in part, before the Medical Session, as part of *Section 3A*, at the annual meeting of the National Tuberculosis Association, New York, New York, May 22, 1956.

TABLE 1
INCIDENCE OF "OPEN-NEGATIVE" DISEASE

Original Treatment		Re-treatment	
per cent		*per cent*	
40	Closed negative	33	Closed negative
40	Open negative	33	Open negative
20	Open positive	33	Open positive
At 6–8 months of chemotherapy		At 6–8 months of chemotherapy	

The classification was essentially clinical and was not modified by subsequent pathologic findings in specimens obtained at surgery or necropsy. Following six to eight months of treatment with antituberculous drugs, these patients were observed to have had sputum cultures negative for tubercle bacilli for at least three consecutive months, yet disclosed persistent annular shadows on planigraphy.

Surgical treatment was indicated for all of these patients because of evidence of persistent cavitation. Nevertheless, 118 of 195 patients did not receive surgical treatment for one or more reasons and will henceforth be termed the nonsurgical group for purposes of follow-up comparisons. A list of the reasons why surgery was not performed follows:

1. Age and unsatisfactory pulmonary function
2. Medically unsanctioned discharge from the hospital
3. Extent of disease
4. Refusal of recommendation for surgery
5. Associated systemic disease
6. Elective thoracoplasty
7. Election of observation

The 5 patients who received primary thoracoplasty had fairly extensive disease and, during the years 1949–1950, pulmonary resection was not being used for this type of disease.

Surgical series: From January, 1951, to December 31, 1954, a total of 153 patients received pulmonary resection on one or both lungs. This group satisfied the bacteriologic criteria of three monthly sputum cultures negative for tubercle bacilli and a roentgenographic estimate of a persistent annular shadow at the time of surgery.

Because of the well-known difficulties in precise interpretation of cavity presence in preoperative planigrams, the terms *equivocal* and *unequivocal* were arbitrarily adopted to describe the estimated state of the "openness" of the lesion. In many instances, it was not possible to be sure that the pretreatment cavity had become a closed lesion. The impression was gained that most observers "overread" the films, erring in the interpretation of an open lesion if the shadows were equivocal. In this study, all planigrams within three months of operation were reviewed and the annular shadows were classified as equivocal or unequivocal (table 2). The possible value of such distinction, and the correlation of preoperative impression with the pathologic findings will be made.

TABLE 2
"OPEN-NEGATIVE" DISEASE
Evidence of Cavity by Preoperative Roentgenography

Original treatment	70 patients
Equivocal open lesion	28
Unequivocal open lesion	42
Re-treatment	83 patients
Equivocal open lesion	30
Unequivocal open lesion	53

TABLE 3
PATIENTS WITH "OPEN-NEGATIVE" SYNDROME SURGICALLY TREATED

Type of operation			
Single or multiple wedges	27	Unilateral	117
Segments (one or more)	105	Bilateral	36
Lobectomy	53	All palpable disease	114 (60%)
Pneumonectomy	4	Residual disease	74
Total	189		

As more than half of the patients with resected "open-negative" lesions came to surgery after receiving various drug combinations in re-treatment regimens, the group was divided to determine the possible influence of the initial and the re-treatment chemotherapy on the immediate and long-term results.

In this series, pulmonary resections were carried out on one or both sides of 153 patients for a total of 189 operative procedures (table 3). Most of the tuberculous lesions in these patients have been discrete enough to allow a segmental resection. Patients whose tubercle bacilli demonstrated some degree of bacillary resistance to one or more drugs prior to "conversion" were regarded as having uncertain drug control at the time of surgery. In these patients (approximately 10 per cent), a lobectomy was the preferred type of resection. Single or multiple wedge resections were utilized whenever feasible, but most often in the patients receiving chemotherapy for the first time. Multiple wedging of residual closed lesions was often supplementary to segmental or lobe resection in an effort to establish a surgically treated group of 114 (60 per cent), in whom all palpable disease had been removed.

Whether only cavity-bearing areas should be removed, whether closed lesions in susceptible segments, i.e., superior segment, lower lobe, should be excised, or whether a patient could be assured a much lower relapse potential if it were possible to remove all palpable disease—all such questions have been uncertain in the experience of the writers, and no current data are available for their solution.

Two patients had an operative injury to the main pulmonary artery, requiring an unplanned pneumonectomy. In 2 other patients, an elective pneumonectomy was completed for an upper lobe cavity and lower lobe bronchiectasis.

In every case, the surgical specimen was examined grossly in the fresh state within twenty-four hours of operation. Careful note was made of the existence and nature of open lesions. In addition, the distribution and average size of adjacent closed lesions were recorded. For the present study, all specimens were reviewed in the fixed state, and, when necessary, were recut to determine the presence of open lesions.

At the time of the first examination of the fresh specimen, material for bacteriologic study was collected as follows: Approximately half of the necrotic material from several closed lesions in a segmental area was removed and pooled for culture and guinea pig inoculation. The segmental bronchi were opened longitudinally and a portion of the wall of the bronchial stump was excised and pooled with swabs of mucus and debris which lay in the lumen of the bronchi. Finally, the cavity or suspected cavity area was opened with clean instruments and an adequate sample of the necrotic material within the cavity was removed, plus a section of tissue from the cavity wall.

RESULTS

Patients Treated Nonsurgically

A group of 118 patients with the "open-negative" syndrome after six to eight months of chemotherapy has been observed for relapse rate without resection.

TABLE 4
TUBERCULOSIS RELAPSE
118 Medically Treated Patients with "Open-Negative" Syndrome

Relapse	Number of Cases	Interval After Observation Point	
Bacteriologic	16	Within one year	37
Roentgenographic	15	Bacteriologic	17
Combined (roentgenographic and bacteriologic)	20	Roentgenographic	11
		Combined	9
Total	51 (43%)		

Only one relapse per patient was counted and the case was then dropped from the analysis as no longer being "at risk" of relapse.

A roentgenographic relapse, which was defined as *any unfavorable roentgenographic change,* occurring after the observation point, was seen in 15 patients without a concomitant bacteriologic relapse (table 4). Bacteriologic relapse, which was defined as evidence of tubercle bacilli, *by either microscopy or culture,* was found in 16 patients not associated with simultaneous roentgenographic activity. The more common pattern of relapse, however, was manifested by both roentgenographic change and the reappearance of tubercle bacilli in the sputum. Such relapses occurred in 20 patients. In many instances, the reappearance of tubercle bacilli in the sputum was the first unfavorable change to be noted. The presence of bacterial resistance to one or more drugs was often apparent at this time.

A total of 51 patients (43 per cent) have experienced relapses of their disease in a follow-up period extending through three post-treatment years. In 29 patients, the relapse occurred while chemotherapy was still being given; in 22, it appeared after the chemotherapy had been stopped. Most of the relapses occurred early: 37 within one year of the observation point and 46 within two years of the observation point.

A comparison of the chemotherapeutic factors in the relapse group with the entire initial treatment group from which they emerged indicates that duration of the chemotherapy was not a significant factor since more than half of the relapses occurred while therapy was still being given. In a retrospective study of factors presumably common or contributing to the relapse group, no definite clinical, bacteriologic, or roentgenographic clues which would permit the forecast of relapse in any particular patient were apparent at the observation point (six to eight months).

Patients Treated with Resection

Surgical complications: Both the immediate and late complications related to the operative procedure are given in table 5. Most of the complications occurred during the first two postoperative weeks and were nontuberculous in origin. In the sense that such complications represent mechanical problems inci-

TABLE 5
SURGICAL COMPLICATIONS
153 Surgically Treated Patients with "Open-Negative" Syndrome

Nontuberculous		Tuberculous	
Pneumothorax	30	Tuberculous reactivation	4
Hemopneumothorax	5	Occult bronchopleural fistula and tuberculous reactivation	4
Bronchopleural fistula	6		
Pneumonia	2		
Operative accidents	2		

dent to pulmonary resection, they were benign, easily treated, and did not delay the patient's ultimate discharge (3).

Even the much discussed bronchopleural fistula, although it may require a small post-resection thoracoplasty for definitive treatment, is not necessarily tuberculous in etiology. As in many resection series, most of these complications occurred during earlier years. A former 20 per cent incidence of transient fistulas has been reduced by certain modifications of technique to a range of 1 to 3 per cent (4).

The major post-resection complications have been seen in a small group of 8 patients, each of whom had a sub-lobar type of resection in the left upper lobe. This surgical morbidity consisted of tuberculous reactivation in the operative site or in the remaining segments of the upper lobe. Because such complications represent not only surgical morbidity but medical treatment failures, as manifested in roentgenographic and bacteriologic relapse, the possible etiologic factors will be discussed in a consideration of the patients who suffered relapse in the entire resected series.

Gross findings in resected specimens: The major findings in the resected specimens have been tabulated (tables 6 and 7). The principal attention in these gross observations has been to determine the existence and nature of open lesions. These have been defined as air-bearing lesions, representing a loss of continuity or area of destruction in the parenchyma. In many instances, the open lesions are recognized as rather typical tuberculous cavities. They often contain variable amounts of inspissated necrotic material. In a small number of specimens, there has been some difficulty in deciding whether a cavity which

TABLE 6
MORPHOLOGY AND BACTERIOLOGY
189 Resected Specimens from Patients with "Open-Negative" Syndrome

	Total	Open Lesions Positive	Open Lesions Negative Closed Lesions Positive	Total Specimens Positive
Open lesions	120	32	17	49 (41%)
Dirty cavities	62	28	11	39 (62%)
Clean cavities	58	4	6	10 (17%)
Closed lesions	69			20 (28%)

has become essentially filled with necrotic debris should be classified as still open or whether it is actually an unsloughed or filled-in closed lesion. By adhering to the criteria of air-bearing, it has been possible to categorize the majority of such lesions.

In the entire group of 189 specimens, a total of 120 specimens (63 per cent) were found to have open lesions (table 6). On the other hand, such lesions were not disclosed in 69 specimens (36.5 per cent) even after careful recutting of the fixed material.

The presence of any amount of necrotic material was sufficient to consider 62 specimens as presenting "dirty" cavities. Most of these lesions were characteristic parenchymal tuberculous cavities. However, 58 of the open lesions were recognized as "clean" cavities. The word cavity is, perhaps, a misnomer, as this group is represented by a variety of pulmonary abnormalities, which appear as facsimiles by roentgenography. Totally sloughed cavities were found to account for approximately one-quarter of the clean open lesions. Several of the open lesions were blebs or bullae and were thought to be a manifestation of focal hypertrophic emphysema adjacent to areas of tuberculosis. A fairly large number of lesions was found to be dilated or cystic bronchi. Whether such bronchial deformity represents a sequela of tuberculous or nontuberculous infection or pre-existent disease acquired on a developmental basis cannot be stated at this time.

The existence and patency of bronchocavitary junctions as a critical factor in possible endobronchial spread were always noted. Again, such a demonstration very often requires confirmation by serial microscopic sections. As such careful micropsy was not available for the entire series, such data are omitted.

The size and distribution of all closed lesions in segments adjacent to the cavity-bearing area were carefully noted. Such lesions are possibly of more importance in their bacteriologic potential than in their physical state which, on the average, was a fairly well-encapsulated fibrocaseous nodule.

Since the only clinical assistance presently available to distinguish the open from the closed negative group is roentgenographic survey, the writers have correlated (table 7) the preoperative impression of an equivocal or unequivocal open lesion with the demonstration of such openness in the resected material. It is evident that, to include a possible open lesion, it was necessary to overread the films or maintain a high index of clinical suspicion. Air-bearing lesions

TABLE 7

PATIENTS WITH "OPEN-NEGATIVE" SYNDROME

Correlation of Roentgenography, Pathology, and Bacteriology

Preoperative Roentgenogram	Number of Cases	Number of Open Lesions	Positive Culture Open or Closed Lesions
Unequivocal cavity	95	79 (83%)	34 (36%)
Equivocal cavity	58	29 (50%)	16 (55%)
Total	153	108 (70.5%)	50 (33%)

were found in more than three-quarters (83 per cent) of resected material from patients with unequivocal annular shadows. However, the group with equivocal preoperative roentgenographic evidence of an open lesion disclosed a lower incidence of cavity (50 per cent) in the operative specimen. These data suggest that a reasonably accurate interpretation can be given to the definition of annular shadows which occurred during treatment.

Specimen bacteriology: The results of bacteriologic study are given in tables 6 and 7. The most important finding is the recovery of viable tubercle bacilli in 69 (38 per cent) of the resected specimens by culture or guinea pig inoculation. This value represents the yield from any lesion in the specimen. In most instances, the organisms were recovered from the open lesions (41 per cent). Occasionally, an adjacent unsloughed or filled-in lesion might yield bacilli when the cavity was bacteriologically negative for tubercle bacilli (14 per cent). In those specimens without any evidence of an open lesion, organisms were recovered in 20 of 69 (28 per cent).

Whenever tubercle bacilli were isolated from the resected material, a determination was made of the resistance or susceptibility of the organisms to the drugs given in the preoperative period.

When drug-susceptible bacilli were found in the "preconversion" sputum cultures, the organism usually exhibited the same drug susceptibility in cultures from the specimen. However, in a certain number (10 per cent) of the "open-negative" patients, various degrees of bacillary resistance may have been evident in "preconversion" sputum cultures.

When "preconversion" drug-resistance was apparent, as a rule, equivalent or greater degrees of drug resistance were shown in the organisms recovered from the original specimen. That such bacillary resistance may progress *in vivo*, while the patient continues in the "sputum-negative" state for three months or longer, is now apparent (5).

Relapse: A total of 13 patients who had had resections were considered to show evidence of tuberculosis relapse (table 8). Five of this group demonstrated either roentgenographic or bacteriologic change, while the remaining 8 exhibited both findings. The 3 patients who had bacteriologic relapse were found, in each instance, to have only a single positive culture that occurred sometime during the one- to six-month interval following surgery. No particular significance was attached to this finding and the planned duration of chemotherapy was not

TABLE 8

TUBERCULOSIS RELAPSE

153 Surgically Treated Patients with "Open-Negative" Syndrome

Relapse		Interval Postoperatively
Operated side (roentgenographic and bacteriologic)	8	2 weeks to 2½ years
Nonoperated side (roentgenographic only)	2	2 months and 6 months
Undetermined (bacteriologic only)	3	1 month to 6 months
Total	13	

changed. There have been no subsequent unfavorable changes in these 3 patients during an eighteen-month follow-up period.

Two patients manifested roentgenographic changes in the nonoperated lung during a six-month interval of postoperative observation. These changes consisted of vacillation in density of equivocal open lesions which had been present prior to the contralateral surgery. For various reasons, excision of these suspect areas was not recommended.

A more dramatic manifestation of tuberculosis relapse was apparent in 8 of the 13 patients. In this group, definitely active exudative disease was seen in the operated lung by serial roentgenograms. The unfavorable changes were usually seen in the remaining segments of the left upper lobe and appeared from two weeks to two years following resection. Following the recognition of disease activity in the chest films, each patient was found to have tubercle bacilli in the sputum.

Further study of these 8 patients by planigraphy, bronchoscopy, and bronchography disclosed in 4 the presence of a pseudo cavity at the site of an apicoposterior segment resection in the upper lobe. Exploration of the lung at secondary thoracotomy revealed an occult fistulous tract of the segmental bronchial stumps. This infected fistulous tract was apparently the source of endobronchial dissemination of tuberculosis into the adjacent lung. The details of pathogenesis, as learned from the gross and microscopic examination of primary and secondary specimens in 8 patients with occult post-resection fistulas (4 of whom showed the "open-negative" syndrome), are described in another report (6).

Of the remaining 4 patients with post-resection reactivation, each had had a segmental resection in the left upper lobe. All 4 exhibited new areas of infiltration in the remaining lingular segments. No cavity or infected operative site was demonstrated by appropriate studies, however, and for various reasons secondary resections were not performed.

An attempt was made to relate these instances of relapse to the nature and duration of pre- and postoperative chemotherapy (table 9*A*). The numbers are small and no conclusions are warranted.

TABLE 9*A*

RELATIONSHIP OF TUBERCULOSIS RELAPSE TO PREOPERATIVE, OPERATIVE, AND POSTOPERATIVE FACTORS

153 Surgically Treated Patients with "Open-Negative" Syndrome

Number of patients with relapse (roentgenographic or bacteriologic)	13
Original treatment	5
Re-treatment	8
Duration of preoperative chemotherapy	
Six months or less	3
More than six months	10
Duration of postoperative chemotherapy	
Less than six months	6
More than six months	7

It is believed that patients being re-treated tend to have more surgical complications than those who receive chemotherapy for the first time Moreover, in these circumstances, the emergence of drug-resistant strains of tubercle bacilli seems to occur more rapidly. Consequently, it is possible that the protection exerted by chemotherapy during and after the resection is not as effective as it is assumed to be. For this reason, it has become the practice on this service to consider surgical intervention at an earlier point in the re-treatment case than in the patient who is receiving chemotherapy for the first time.

Postoperative chemotherapy, if it is maintained for a minimum of six months, appears to be unrelated to the incidence of relapse. Considering the type of surgical procedure, relapse occurred most often following sub-lobar resection. This is, perhaps, related to the greater opportunity for implantation infection consequent to segmental resections. Each of the combined roentgenographic and bacteriologic relapses (8 patients) followed bisegmental or trisegmental resection in the left upper lobe. Two of the tuberculous reactivations were seen in patients who had had all palpable disease removed at a primary operation.

One of the most interesting findings was the relationship of the microbiologic findings in the resected specimen to the possibility of subsequent relapse (table 9*B*). Seven of the 13 patients had resected material which was negative for tubercle bacilli on culture. In 2 of these 7 patients, the unfavorable roentgenographic changes occurred in the side not operated upon and, hence, were

TABLE 9*B*

RELATIONSHIP OF TUBERCULOSIS RELAPSE TO PREOPERATIVE, OPERATIVE, AND POSTOPERATIVE FACTORS

153 Surgically Treated Patients with "Open-Negative" Syndrome

Number of patients with relapse (roentgenographic or bacteriologic)	13		
Surgical procedure		Specimen bacteriology	
Multiple wedges	2	Negative culture	7
Segments	8	Positive culture	6
Lobe and wedges	3	Susceptible	2
All palpable disease	6	Resistance to one or more drugs	4
Residual disease (ipsilateral side)	7		

TABLE 9*C*

PATIENTS WITH "OPEN-NEGATIVE" SYNDROME

Relationship of Relapse to Culture of Specimen and Residual Unresected Closed Lesions

	Number of Cases	Number of Relapses	Per Cent of Relapses
Specimen negative	94		
All palpable disease	59	3	5.0
Disease remaining	35	5	14.0
Specimen positive	59		
All palpable disease	31	2	6.4
Disease remaining	28	3	10.0

presumably unrelated to the bacteriologic status of the lesion which had been subjected to resection.

Three additional patients whose resected specimens were negative on culture for tubercle bacilli exhibited single sputum cultures positive for tubercle bacilli during the postoperative period. However, closed lesions up to 1 cm. had been left at the time of resection. Since the bacteriologic relapse was not associated with roentgenographic activity, the site of relapse could not be identified. The remaining 2 patients, although showing specimens negative for tubercle bacilli, had residual nonresected closed lesions which presumably were the source of the relapse.

Of the 6 patients who had resected material which yielded viable organisms, in 4 the cultures showed resistance to one or more drugs *in vitro*. The presence of such resistant bacilli in the tissues at the time of resection is tantamount to operating without effective drug control. Although the results of the tissue cultures were not known during the actual progress of the surgery, a subsequent study of the "open-negative" group revealed that such findings could be anticipated when drug-resistant bacilli appeared in routine cultures prior to sputum "conversion."

Follow-up Observations

Patients treated nonsurgically: Follow-up observations of the patients treated nonsurgically (118 cases) have been quite complete: Of those at risk of relapse, follow-up observations for one year were available on 96 per cent; for two years on 92 per cent; and for three years on 86 per cent.

The current status of the medical or nonsurgical group is presented in table 10*A*. Of the 90 patients (76 per cent) now considered to have inactive disease, 67 never demonstrated relapse. In the remaining 23 patients, relapse occurred at various intervals from the observation point (six to eight months), but the disease was converted to an inactive status by continuing the original chemotherapy, by re-treatment with or without a change of the chemotherapy, and by subsequent pulmonary resection.

Fifteen (12 per cent) of the original group are still considered to have active disease, and most of these patients are still in the hospital. There were 8 deaths from tuberculosis and 5 from nontuberculous causes.

Patients treated with resection: The current status of the group treated with

TABLE 10*A*
CURRENT STATUS
118 Medically Treated Patients with "Open-Negative" Syndrome

Inactive	90 (76%)	Active	15 (12%)
Never relapsed	67	Deceased	13
Relapsed but inactive from	23	Tuberculosis	8
1. Continuing treatment		Nontuberculous causes	5
2. Re-treatment chemotherapy			
3. Surgery			

TABLE 10*B*
CURRENT STATUS
153 Surgically Treated Patients with "Open-Negative" Syndrome

Inactive	133 (87%)
Active	6 (4%)
Deceased (nontuberculous causes)	4
Lost to follow-up observation	10

resection is given in table 10*B*. The last patient included in this review was operated upon in December, 1954. Thus, a minimum of one-year, and, for the majority, of two- and three-year records of follow-up observations are available. Ten patients were lost to the study in spite of repeated efforts to trace them. There were no postoperative deaths, and the 4 deaths in the series were from nontuberculous causes. A total of 133, or 87 per cent of the original 153 patients, are classified as having inactive tuberculosis. Four are receiving re-treatment chemotherapy. The site of disease activity is believed to be in the operated lung.

DISCUSSION

This survey was undertaken to compare the current results of the medical or medical and surgical treatment of the tuberculous patient with persistent cavitation despite sputum negative for tubercle bacilli. Because this group comprises a considerable number of the hospital population, the problem is perhaps more real than the ultimate fate of the patient whose cavities have closed under chemotherapy.

The early experience of the writers with patients with the "open-negative" syndrome suggested that this clinical status was frequently followed by relapse (7). In at least half of the early "open-negative" group (18 of 35 patients), relapse occurred while the patient was still on treatment or within the first year after treatment had been stopped.

The problem is to identify beforehand the half with the relapse potential. Lacking roentgenographic criteria to make what is, in essence, a pathologic distinction, in the present series it was assumed that there was a 50 per cent risk of relapse for the group as a whole. Accordingly, surgical resection was recommended for all patients with the "open-negative" syndrome who were reasonable surgical risks.

More recently, an attempt has been made to refine the criteria for selection of cases for resection. The almost routine use of preoperative bronchograms has helped considerably in correlating the appearance of residual annular shadows with the morphology of the resected specimen.

Consideration of the follow-up data, as the nonsurgical group (118 patients) increases, reveals that there is still a significant incidence (43 per cent) of relapse in patients who show the "open-negative" syndrome. The fact that, in 29 cases, clinical relapse occurred while the patients were still receiving chemotherapy and, in 37, within one year of the six- to eight-month observation point, suggests that the patient with the "open-negative" syndrome is at a fairly constant risk

of reactivation of his disease, provided that the "open" status persists. The eventual achievement of an inactive disease status in 90 of 118 patients (76 per cent) is explained by the possibility of altering the relapse pattern. Thus, with the demonstration of an unfavorable roentgenographic development, either a change in drugs, pulmonary resection, or both, may be advised without waiting for possible bacteriologic relapse.

Or the contrary may occur with the reappearance of tubercle bacilli in the sputum as the signal for a change in chemotherapy or surgery, if the latter is feasible. Thus, in 23 patients, whose disease relapsed after the six- to eight-month observation point, the lesions were influenced (table 10*A*) by various combinations of therapy and were brought to an inactive status. At the present time, there appears to be no common denominator for prediction of those patients whose disease will subsequently relapse.

In view of this, an effort has been made to advise resection for all patients with the "open-negative" syndrome who are reasonable surgical risks. The absence of operative mortality and the low incidence of tuberculous surgical complications (2 per cent) seem to indicate that such pulmonary resection is essentially safe. The mere fact of being safe is no justification for perpetuating such a surgical program unless the outlook of an individual patient can be significantly improved.

The current status of the resected "open-negative" group, 133 of 155 patients (87 per cent) having inactive disease when last observed, suggests that pulmonary resection has a good deal to offer toward influencing the prognosis.

It is probable that application of current knowledge in surgical technique and the careful timing of resection with the use of appropriate new drugs will improve the surgical results still further.

Summary

A comparison has been made of the results in 153 patients treated surgically and in 118 patients treated nonsurgically, all of whom displayed the "open-negative" syndrome of pulmonary cavitation persisting after the disappearance of tubercle bacilli from the sputum.

The nonsurgical group was composed entirely of patients who received antituberculous chemotherapy for the first time. The surgical group was composed of approximately half previously untreated and half previously treated patients. No essential difference attributable to re-treatment was apparent provided the patient was receiving an effective combination of drugs at the time of surgery.

The problem of selecting at the six- to eight-month observation point the patients who will eventually display the "open-negative" syndrome is aided by classifying the findings on serial planigraphy as "equivocal" or "nonsurgical." When an unequivocal annular shadow was present preoperatively, an open lesion was found in 87 per cent of the gross specimens. The accuracy of prediction was reduced to 50 per cent in the group judged to have "equivocal" findings.

Viable tubercle bacilli were recovered from nearly half of those specimens which showed recently filled-in cavities in the areas thought to be open lesions.

The recovery of viable tubercle bacilli from open or closed lesions in 38 per cent of the specimens and a relapse rate of 43 per cent in the nonsurgical series emphasized the failure of drug therapy to eradicate all tubercle bacilli from the lesions, thus providing a focus for potential relapse. Nevertheless, in the surgical group, relapse was as common in those with resected specimens containing no tubercle bacilli as in those whose resected specimens contained culturable tubercle bacilli (table 9C). In the patients with significant unresected "closed" disease, either on the resected or non-resected side, the hazard of relapse was greater than in those in whom it was possible to remove virtually all palpable tuberculous disease (60 per cent).

In patients with the "open-negative" syndrome, the major relapse factor in those treated nonsurgically appears to be the residual annular lesion. In the surgical group, the presence of known or unsuspected drug-resistant bacilli at the time of surgery plus the inability to remove all palpable disease, either unilateral or bilateral, are the predominant factors in the occurrence of relapse.

Relapse rates of 43 per cent and 8.4 per cent for the nonsurgical and surgical groups, respectively, indicate that pulmonary resection is valuable in improving the over-all results in patients with the "open-negative" syndrome.

Sumario

Comparación de los Resultados del Tratamiento Quirúrgico y No Quirúrgico de Tuberculosos Que Muestran el Síndrome "Abierto-Negativo"

Trázase una comparación de los resultados obtenidos en 153 tuberculosos tratados quirúrgicamente y en 118 tratados incruentamente, todos los cuales mostraban el síndrome "abierto-negativo" de cavitación pulmonar persistiendo después de desaparecer los bacilos tuberculosos del esputo.

El grupo incruento constaba totalmente de enfermos que habían recibido quimioterapia antituberculosa por primera vez. El quirúrgico estaba compuesto aproximadamente a medias de enfermos tratados y no tratados antes. No se notaba ninguna diferencia esencial imputable al re-tratamiento, siempre que el enfermo recibiera una combinación eficaz de drogas al ejecutarse la cirugía.

Simplifícase el problema de seleccionar, en el punto de observación de seis a ocho meses, a los enfermos que mostrarán con el tiempo el síndrome "abierto-negativo" clasificando los hallazgos de la planigrafía seriada como "equívocos" o "no quirúrgicos." Cuando existía preoperatoriamente una sombra anular inequívoca, se encontró una lesión abierta en 87 por ciento de los ejemplares macroscópicos. La exactitud de la predicción descendió a 50 por ciento en el grupo en que se juzgó que había hallazgos "equívocos."

Obtuviéronse bacilos tuberculosos viables casi de la mitad de los ejemplares que mostraban cavernas recién henchidas en las zonas consideradas como lesiones abiertas. La obtención de bacilos tuberculosos viables de lesiones abiertas o cerradas en 38 por ciento de los ejemplares y un índice de 43 por ciento de recidivas en la serie incruenta hicieron resaltar el fracaso de la farmacoterapia en lo tocante a erradicar todos los bacilos tuberculosos de las lesiones, creando así un foco de recidiva potencial. No obstante, en el grupo quirúrgico, las recidivas fueron tan frecuentes en aquéllos cuyos ejemplares resecados no contenían bacilos tuberculosos como en aquéllos cuyos ejemplares resecados contenían bacilos tuberculosos cultivables. En los sujetos que tenían enfermedad "cerrada" resecada de importancia, ya en el lado resecado o el otro, el riesgo de recidiva era mayor que en aquéllos en que era posible extirpar virtualmente toda la enfermedad tuberculosa palpable (60 por ciento).

En los enfermos del síndrome "abierto-negativo," el factor mayor en la recidivación de

los tratados incruentamente parece ser la lesión anular residual. En el grupo quirúrgico, la presencia conocida o insospechada de bacilos fármacorresistentes al llevar a cabo la cirugía y además el incumplimiento de la extirpación de toda la enfermedad palpable, ya uni- o bilateral, son los factores predominantes en la recidivación.

Los índices de 43 por ciento y 8.4 por ciento de recidivación en los grupos no quirúrgico y quirúrgico, respectivamente, indican que la resección pulmonar resulta valiosa para mejorar los resultados globales en los enfermos del síndrome "abierto-negativo."

Resume

Etude comparative des résultats du traitement chirurgical et non chirurgical des tuberculeux pulmonaires porteurs de cavernes épithélialisées et abacillifères ("open negative" syndrome)

Les résultats du traitement chirurgical chez 153 malades et non chirurgical chez 118 malades porteurs de cavernes épithélialisées avec négativation des crachats sont comparés.

Le groupe non chirurgical se composait entièrement de malades soumis pour la première fois à la chimiothérapie. Le groupe chirurgical comprenait, en proportion sensiblement égale, des malades antérieurement non traités et des malades déjà traités. Aucune différence essentielle imputable au "re-traitement" n'a été décelée, à condition que le malade ait été soumis à une thérapeutique combinée efficace au moment de l'intervention chirurgicale.

Le problème de la sélection des malades susceptibles de présenter des cavernes épithélialisées avec négativation de l'expectoration (open negative syndrome), au terme d'une mise en observation de six à huit mois, est facilité par la classification des aspects de la planigraphie en série en deux catégories: "équivoque" ou "non chirurgicale." Lorsqu'une opacité annulaire non équivoque était présente avant l'intervention, l'examen macroscopique révélait une excavation épithélialisée dans 87% des pièces réséquées. L'exactitude des prévisions était réduite de 50% dans le groupe dont les aspects planigraphiques étaient considérés "équivoques."

Des bacilles tuberculeux viables étaient récupérés dans presque la moitié des pièces où il y avait des cavernes récemment oblitérées situées dans des zones considérées comme des lésions ouvertes. L'isolement de bacilles tuberculeux viables à partir de lésions ouvertes ou fermées dans 38% des pièces de résection, et un taux de rechute de 43% dans le groupe non chirurgical, confirment l'échec de la thérapeutique médicamenteuse pour l'éradication de tous les bacilles des lésions, ce qui constitue un facteur potentiel de rechute. Toutefois, dans le groupe chirurgical, les rechutes étaient aussi fréquente chez les sujets dont les pièces de résection ne renfermaient pas de bacilles que chez ceux où les pièces réséquées contenaient des bacilles tuberculeux cultivables. Chez les malades porteurs de lésions "fermées" significatives, du coté réséqué ou non réséqué, le risque de rechute était plus grand que chez ceux où il était possible de réséquer virtuellement toutes les lésions tuberculeuses décelables (60%).

Chez les sujets présentant le syndrome "open-negative," le facteur principal de la rechute de cas traités non chirurgicalement paraît être la lésion annulaire résiduelle. Dans le groupe chirurgical, la présence de bacilles à résistance connue ou inconnue et le moment de l'intervention ainsi que l'impossibilité d'exciser toutes les lésions décelables, unilatérales ou bilatérale, constituent les facteurs prédominants des rechutes.

Les taux de rechute de 43% pour le groupe non chirurgical et de 8,4% pour le groupe chirurgical indiquent la valeur de la résection pulmonaire pour améliorer l'ensemble des résultats chez les malades porteurs de cavernes épithélialisées et abacillifères ("open-negative" syndrome).

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CYCLOSERINE COMBINED WITH OTHER ANTITUBERCULOUS AGENTS IN THE TREATMENT OF PULMONARY TUBERCULOSIS[1,2,3]

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INTRODUCTION

A number of independent investigators agree that cycloserine, the low molecular weight antimicrobial derived from *Streptomyces orchidaceous* (1, 2), has useful antituberculous properties in man (3–5, 7, 20). In oral doses of 1.0 gm. per day, patients with long-standing, drug-resistant, pulmonary tuberculosis responded well (6–10) and mortality was nil during the first year of treatment. A similar but concomitant cycloserine-untreated group showed a mortality of 23 per cent during the same period (8, 9). The present investigators were greatly encouraged by these results, despite the occurrence of convulsive seizures in 6 per cent of the patients. The clonic manifestations were not severe, and were never injurious. When they did occur, cycloserine was discontinued entirely, or the dose was temporarily reduced to 0.75 or 0.5 gm. per day and there was no further difficulty. At any rate, neither the frequency nor the severity of the seizures precluded trial of cycloserine in the hopeless cases of tuberculosis. The physicians in the U. S. Veterans Administration have reported similar results (7).

Following proof of the efficacy of cycloserine alone against pulmonary tuberculosis, additional studies were instituted, using smaller doses of cycloserine, in an attempt both to define the effective dose and to reduce the incidence of side reactions. In the first study the dose of cycloserine was reduced to 0.5 gm. per day when given alone to a series of 30 patients. These patients were part of a group with resistant tuberculosis, treated at Metropolitan Hospital in New York City, who had not received cycloserine during the original study. They received 0.25 gm. of cycloserine twice a day. Their progress during the year was compared with that in patients who had received 1.0 gm. of cycloserine per day. The respective clinical responses will be reported elsewhere. There were no toxic manifestations in this group during the following six months of treatment.

In the second study, the results of which are reported below, doses of 0.5 or 1.0 gm. of cycloserine were used daily, and either oral isoniazid daily or intramuscular streptomycin biweekly.

PLAN OF STUDY

This report gives the details of the clinical effects so far observed with cycloserine in combination with other drugs. Patients were not selected in any way, but were put on the

[1] From the Department of Medicine, New York Medical College; and the Tuberculosis Division, Metropolitan Hospital, New York, New York.

[2] Cycloserine is manufactured by Commercial Solvents Corporation, Terre Haute, Indiana, and marketed by Eli Lilly and Company, Indianapolis, Indiana, as Seromycin (Cycloserine, Lilly).

[3] Presented before the 22nd annual meeting of the American College of Chest Physicians, Chicago, Illinois, June 10, 1956.

chosen regimen upon admission to the wards of Metropolitan Hospital. No patients with minimal tuberculosis happened to have been admitted to the service during the period of the study. All of the patients had tubercle bacilli in the sputum on microscopy and culture. All gave a typical history of productive cough and loss in weight and strength. The roentgenographic designation of the degree of pulmonary involvement was that of the National Tuberculosis Association. The degree of pulmonary involvement and the other relevant data for each group of cases are shown in the tables. With but one exception, no collapse or excisional measures were used. The cases are divided into two groups.

Drug Regimens

Group I. Cycloserine and isoniazid combined therapy: Two dosage levels of cycloserine and isoniazid were used: cycloserine, 1.0 gm., and isoniazid, 300 mg. (*Group IA*); and cycloserine, 0.5 gm., and isoniazid, 4.0 mg. per kg. (*Group IB*). Both drugs were given daily by the oral route.

Group II: Cycloserine, 1.0 gm. per day, orally, and streptomycin, 1.0 gm. biweekly, intramuscularly.

In reporting the results of treatment, especially the roentgenographic findings, the following terms have been used:

Marked clearing: No, or only a very slight amount of residual abnormality, in which tomography shows no evidence of previously demonstrable cavitation.

Moderate clearing: Less clearing than above, but the extent of resolution represents at least half of the abnormal densities previously present.

Slight clearing or no change: All cases which show less than moderate clearing or have remained stationary without regression.

Worse: Includes the development of a new lesion or an increase of cavity size even though there may have been some resolution on a previous film. Chest films were obtained at least every four weeks in order to follow closely the rate of resolution.

The data detailed in the tables include observations made after six weeks of treatment in order to show the promptness of response to the isoniazid-cycloserine combination.

In reporting sputum "conversion," the data in all cases include sputum and gastric aspirates negative for tubercle bacilli on both smear and culture.

Results

Therapeutic Effects

Data were obtained on 43 subjects with moderately to far advanced pulmonary tuberculosis who were treated with cycloserine and isoniazid combinations. In table 1 the pertinent information is shown on these subjects prior to the institution of therapy. The pulmonary lesions were moderately advanced in 28 of the patients and far advanced in the remaining 15. The results from the use of the larger doses of cycloserine and isoniazid were about equal to those from the smaller dosage combinations. Accordingly, the results in the subgroups (*IA* and *IB*) are reported separately because of the virtual absence of toxic side effects from the smaller doses of cycloserine.

Group IA consisted of 13 adult subjects, 8 females and 5 males, between the ages of twenty-four and forty-nine years (average thirty-one years). Each subject received orally 0.25 gm. of cycloserine four times a day and 150 mg. of isoniazid twice a day. The results are shown in tables 2 to 4. In agreement with previous experience (6), there was prompt symptomatic improvement and defervescence within a week or two after the start of therapy. Within six weeks all 13 subjects gained in weight, as shown in table 2. As may be seen in table 3, there

was significant roentgenographic improvement in a majority of the patients within six weeks of institution of therapy. Three patients had improved sufficiently to leave the hospital (against medical advice) after ten to sixteen weeks of therapy.

The speed and incidence of sputum "conversion" or reversal of infectiousness (11) corroborate fully the results obtained on analysis of the roentgenographic changes. In all of the 22 patients in *Group I*, tubercle bacilli had disappeared from the sputum, by both microscopy and culture, by the twenty-fourth week of treatment (table 4).

TABLE 1

ISONIAZID-CYCLOSERINE COMBINATION THERAPY IN PULMONARY TUBERCULOSIS

Group I

Age: 17 to 57 years (average, 32 years)

Sex: 19 female 24 male

Race: 15 Negro 17 Puerto Rican

9 white 2 other

Initial Pulmonary Pathology on Roentgenography

(No Minimal Cases Used)

Type of Lesion	Stage of Lesion*				
	Far Advanced		Moderately Advanced		Total
	IA†	IB‡	IA	IB	
Caseo-pneumonic	2	6	2	4	14
Cavitary	1	6	6	9	22
Fibrocaseous	0	0	2	5	7
	3	12	10	18	43

* According to the 1950 classification of the National Tuberculosis Association.

† 1.0 gm. of cycloserine and 300 mg. of isoniazid per day for 6 to 48 weeks

‡ 0.5 gm. of cycloserine and 4.0 mg. of isoniazid per kg. per day for 6 to 28 weeks

TABLE 2

ISONIAZID-CYCLOSERINE COMBINATION THERAPY IN PULMONARY TUBERCULOSIS

Group I

Body Weight Change During Treatment

Initial Weight, All Subjects: 73 to 193 Pounds (Average, 118 pounds)

Weeks of Treatment	Number of Cases		Weight Change in Pounds		
			Average		Range
	IA	IB	IA	IB	IA and IB
6	13	30	8.1	7.9	−5 to +21
12	12	20	16.5	16.7	0 to 32
24	10	12	28.0	29.0	14 to 41
36	9	—	33.1	—	19 to 48
48	5	—	28.2	—	19 to 41

Symptomatic improvement was prompt in all cases.

TABLE 3

ISONIAZID-CYCLOSERINE COMBINATION THERAPY IN PULMONARY TUBERCULOSIS

Group I

Roentgenographic Changes

Weeks of Treatment	Degree of Change											
	Marked Clearing			Moderate Clearing			Slight to No Change			Worsening		
	IA	IB	IA and IB	IA	IB	IA and IB	IA	IB	IA and IB	IA	IB	IA and IB
6	0/13	1/30	1/43	9	17	26	3	10	13	1	2*	3
12	6/11	7/20	13/31	4	11	15	1	2	3	0	0	0
24	7/10	8/12	15/22	3	4	7	0	0	0	0	0	0
36	8/9	—	8/9	1	—	1	0	—	0	0	—	0
48	4/5	—	4/5	1	—	1	0	—	0	0	—	0

* One of these developed a spontaneous pneumothorax at the fourth week, but treatment was continued.

TABLE 4

ISONIAZID-CYCLOSERINE COMBINATION THERAPY IN PULMONARY TUBERCULOSIS

Group I

Bacteriologic Status

Initially all sputum was positive for *M. tuberculosis* on smear and culture.

Weeks of Treatment	Sputum* Negative			
	Smear		Culture	
	IA	IB	IA	IB
6	7/13	22/30	2	12
12	12/12	20/20	11	16
24	10/10	12/12	10	12
36	9/9	—	9	—
48	5/5	—	5	—

* Includes sputum and gastric washings.

Side reactions from the combination of cycloserine and isoniazid did not increase over those due to cycloserine alone.

The *Group IB* cases were treated with reduced doses of both cycloserine and isoniazid. The data suggest a degree of effectiveness against pulmonary tuberculosis which is superior to that seen when either drug is used alone. The results shown in tables 2 to 4 are similar to those of *Group IA*, although they were obtained with the regimen of 0.5 gm. of cycloserine and 4.0 mg. of isoniazid per kg. per day. Rate of weight gain, reversal of infectiousness, and significant roentgenographic improvement were about the same in these two isoniazid-cycloserine subgroups. This may indicate that the daily dose of 1.0 gm. of cycloserine combined with isoniazid, as used in *Group IA*, was in excess of that needed for optimal effects.

In *Group II* the patients received cycloserine and streptomycin. In tables 5 to 8 are summarized the data from 14 patients whose clinical parameters were

TABLE 5

STREPTOMYCIN-CYCLOSERINE COMBINATION* THERAPY IN PULMONARY TUBERCULOSIS

Group II

Age: 18 to 48 years (average, 31 years)

Sex: 3 female 11 male

Race: 5 Negro 7 Puerto Rican 2 white

Initial Pulmonary Pathology on Roentgenography

(No Minimal Cases Used)

Type of Lesion	Stage of Lesion		
	Far Advanced	Moderately Advanced	Total
Caseo-pneumonic	1	2	3
Cavitary	5	4	9
Fibrocaseous	1	1	2
	7	7	14

* 1.0 gm. of cycloserine per day, orally, and 1.0 gm. of streptomycin twice weekly, intramuscularly

TABLE 6

STREPTOMYCIN-CYCLOSERINE COMBINATION THERAPY IN PULMONARY TUBERCULOSIS

Group II

Body Weight Change During Treatment

Initial Weight, All Subjects: 62 to 145 Pounds (Average, 125 Pounds)

Weeks of Treatment	Number of Cases	Weight Change in Pounds	
		Average	Range
6	14	9.1	−10 to +25
12	14	14.6	−3 to +35
24	10	23.9	6 to 46
36	8	31.0	11 to 51

Symptomatic improvement was prompt in all cases.

similar to those of the isoniazid-cycloserine group (table 2). Significant roentgenographic improvement occurred in all of the 14 patients. Nevertheless, as may be seen in table 8, the progress in this streptomycin-cycloserine group was not as rapid as it was in the isoniazid-cycloserine group (table 3). Furthermore, sputum "conversion" was not as rapid (table 7) as it was with the isoniazid-cycloserine regimens (table 4). One male patient had gained 25 pounds during the thirty-six weeks of treatment, but showed a residual cavity, and tubercle bacilli could be cultured from the sputum. This is the only one of the 57 patients in the present study in whom excisional therapy was judged to have been necessary.

Side Effects

The side effects observed during the long-term administration of cycloserine have been described elsewhere (6, 8, 10). The reactions so far ascribed to the drug have been almost exclusively of neurologic origin. After more than a year of use

TABLE 7

STREPTOMYCIN-CYCLOSERINE COMBINATION THERAPY IN PULMONARY TUBERCULOSIS
Group II
Bacteriologic Status
Initially all sputum was positive for *M. tuberculosis* on smear and culture.

Weeks of Treatment	Number of Cases	Sputum* Negative	
		Smear	Culture
6	14	9	3
12	14	11	5
24	10	9	6
36	8	8	7

* Includes sputum and gastric washings.

TABLE 8

STREPTOMYCIN-CYCLOSERINE COMBINATION THERAPY IN PULMONARY TUBERCULOSIS
Group II
Roentgenographic Changes

Weeks of Treatment	Number of Cases	Degree of Change			
		Marked Clearing	Moderate Clearing	Slight to No Change	Worsening
6	14	0	4	10*	0
12	13	0	7	6	0
24	10	3	4	3	0
36	7	3	4	0	0

* One of these patients signed out prior to twelfth-week chest film, but is included in sputum and body weight data (tables 6 and 7).

in many patients, cycloserine alone, in full daily doses, has induced no toxic effects upon the liver, kidney, or the hematopoietic system (12).

The long-term administration of large doses of cycloserine has resulted in the following neurologic symptoms: tremors and hyperreflexia; clonic convulsions, usually of a nonrecurrent, momentary character and often preceded by a period of tremors and hyperreflexia; psychotic changes which have occurred more frequently in patients with a psychotic background (4, 6, 8, 19).

The use of a daily dose of 0.5 gm. of cycloserine with isoniazid in the present study has resulted in a single nocturnal clonic convulsive movement in one patient. The drug regimen has been continued in this patient without further evidence of neurotoxicity.

DISCUSSION

The primary purpose of the present investigation was to discover the dose of cycloserine in combination with isoniazid or streptomycin which would yield the most satisfactory therapy for pulmonary tuberculosis with a minimum of toxicity. It had been shown previously that cycloserine alone represented effective therapy

in tuberculosis (8). However, as is the case with other antimicrobial agents used alone, the occurrence of side reactions from full doses limited the usefulness of cycloserine as the sole therapy. In the treatment of chronic, resistant, "hopeless" cases on the writers' service, the use of cycloserine has reduced the mortality from tuberculosis from 23 per cent to nil during a year of observation.

It is recognized that the number of cases so far studied is relatively small. It is also hazardous to compare results on other regimens, such as isoniazid-PAS, especially when these data are from other institutions. However, it is tempting to speculate on the future of isoniazid-cycloserine therapy on the basis of the superior results so far obtained. It may be that the concomitant administration of isoniazid was largely responsible for the salutary effects observed and that the presence of cycloserine was merely supportive. It is the opinion of the writers that this is not the case, based on their own experience with isoniazid alone or isoniazid-PAS and on the published data of others.

In the present study, at the twenty-four-week observation point, 22 of 29 patients (76 per cent) had shown marked or moderate roentgenographic improvement in contrast to the forty-week value of 50.6 per cent on isoniazid alone in the U. S. Public Health Service study (16*b*). In the study by Deuschle and associates (17) on isoniazid alone at the twenty-four-week observation point, i.e., six months, marked or moderate roentgenographic improvement was observed in 24 of 43 patients (56 per cent), an additional 4 having been dropped from the study. It should be noted that the case material in the present study is not strictly comparable with that of the study by Deuschle and associates in that 22 of their 47 patients had had previous therapy with streptomycin. In their group of 47 patients with pulmonary tuberculosis, 32 finished a full year of therapy. At this time, 21 showed substantial roentgenographic improvement, i.e., marked and moderate combined. Four became worse, in addition to 11 of the 47 subjects who had been eliminated previously because of drug resistance, lack of improvement, or the necessity for collapse or excisional procedures. Eleven of the 32 patients showed positive cultures for tubercle bacilli after a year on isoniazid alone. On the valid assumption that the 11 patients who were not helped would have continued to discharge tubercle bacilli, the incidence of reversal of infectiousness after a full year of treatment with isoniazid would have been approximately 50 per cent. In table 9 data are presented from various studies on the effectiveness of isoniazid alone in the treatment of pulmonary tuberculosis and are compared with the present data on the isoniazid-cycloserine and streptomycin-cycloserine combinations. The superiority of the isoniazid combinations over isoniazid alone is quite obvious at the twelve-week observation point.

The carefully detailed studies of the Veterans Administration reported by Tucker and Livings (11), using isoniazid-PAS, offer a better basis for comparison because this appears to be the regimen in vogue at the present time. The Tucker-Livings most favorable data on sputum "conversion" (11*a*) for all types of cases which was obtained with isoniazid-PAS combinations may be compared with the present data. The percentile response obtained on sputum "conversion" with the isoniazid-cycloserine regimen in the treatment of 43 moderately to far advanced

TABLE 9

VARIOUS REGIMENS
PULMONARY TUBERCULOSIS
12-Week Data

Investigator	Roentgenographic Changes			Sputum Conversion		
	Number of Cases	Significant Improvement		Number of Cases	Negative on Culture	
		Number	Per Cent		Number	Per Cent
A. Deuschle *et al.* (17)	47	21	45	47	24	50
B. M.R.C. report (18)	129	26	22	101	37	37
C. Isoniazid-cycloserine (present study)	31	28	90	32	27	84
D. VA Report (11*a*)	724	319	44	703	364	50

A. Isoniazid alone, 5.0 mg. per kg. per day: Ref. No. 17.
B. Isoniazid alone, 200 mg. per day: Ref. No. 18.
C. Isoniazid and cycloserine: *see* table 1.
D. Isoniazid-PAS combinations for moderately advanced cases after 17 weeks of treatment: Ref. No. 11*a*.

cases was more favorable than the results reported in the much larger series (500 cases) treated with isoniazid-PAS in the Tucker-Livings report. In the latter study, 100 per cent sputum "conversion" was obtained only in minimal cases after a year of treatment, in contrast to the results with isoniazid-cycloserine in the present study. Clinical response, rate of weight gain, and roentgenographic improvement are also in keeping with the rates of sputum conversion (table 9).

In the present study, after six to forty-eight weeks of treatment with isoniazid-cycloserine combinations, and irrespective of how soon the improvement was "significant," the majority of patients had significant roentgenographic changes, paralleling the findings on sputum "conversion." Furthermore, there were no unfavorable roentgenographic changes in the 49 patients treated for more than six weeks, 29 of whom were continued under treatment for more than twenty-four weeks. Moreover, once the sputum had become negative for tubercle bacilli on culture, bacteriologic relapse was not observed during the twenty-four-week period or later in the 9 patients who were treated for longer periods.

As further corroboration of the marked degree of response, 4 subjects may be cited who, after twenty-seven to thirty weeks of treatment with 0.5 gm. of cycloserine and 4.0 mg. of isoniazid per kg. daily, showed almost complete clearing of the lung fields (figures 3, 4, and 5). The sputum had become negative for tubercle bacilli on smear and culture by the twenty-fourth week in all cases.

The concept in support of multiple-drug therapy is based upon a hoped for reduction in toxicity of the individual drugs and on an additive effect against the bacteria. An organism weakened by one drug may readily succumb to another if the other is present at the same time. The superiority of *in vivo* over *in vitro* effects is ascribed to the capacity of the normal defensive mechanisms of the tissues to finish the destruction of drug-weakened bacteria. This is the reason why drugs

which are only weakly active *in vitro* might become highly effective agents when given to patients.

When a combination of drugs has an effect which is superior to that which can be attained with optimal doses of either one alone, an enhancement or potentiation of action, or "synergism," is said to have occurred. In tuberculosis, speed of reversal of infectiousness has obvious advantages, both hygienic and economic, which are highly desirable. In the present limited series of observations with a combination of two potent antituberculous agents, cycloserine and isoniazid, goals of speed, degree of response, and final over-all recovery rates have been achieved, which, to our knowledge, have not been previously achieved with any other single- or multiple-drug therapy. Furthermore, this high degree of response was obtained on a group of previously untreated, recent cases of tuberculosis, without the necessity of collapse or excisional procedures or change in chemotherapy in any case. What the results will be in more chronic cases, especially in patients previously treated, cannot be surmised at this writing.

It it stressed at this time that, because the isoniazid-cycloserine combination gave quicker and better results, a potentiation of action against the tubercle bacillus must have occurred. These superior results can be ascribed specifically to the treatment rather than to the salutary reactivity of the group of patients, as demonstrated by a similar group studied concurrently but receiving a streptomycin-cycloserine regimen. The patients in this group responded well, as was to be expected, but there was no evidence of potentiation.

In this connection one may cite the observations of Schmidt (13) made on tuberculous monkeys which point to a synergistic action between cycloserine and isoniazid. In addition, Schmidt reports a number of cases in which the tuberculous lesions in the monkeys cleared completely without fibrotic residuum. This may be an explanation for the clearing of the lung fields in 4 of the present cases, as described above. Synergism *in vitro* has been shown to occur between cycloserine and isoniazid (14).

The streptomycin-cycloserine combination gave definitely less favorable results than the isoniazid-cycloserine regimen. This observation, too, has its counterpart in Schmidt's findings on monkeys. While he could obtain no additive effect with streptomycin-cycloserine combinations in his monkeys, his data also suggested that there might even be a slight interference between the two drugs.

Bacterial resistance to cycloserine develops slowly, both *in vitro* and clinically. It is assumed that the isoniazid-cycloserine combination therapy presently employed has delayed or eliminated the development of bacillary resistance to both cycloserine and isoniazid. At any rate, sputum "conversion" has been very rapid. The sputum became negative for tubercle bacilli by the twelfth week of treatment in 27 of the 32 patients (84 per cent) on whom data are available. Treatment in many cases has been continued much longer than thirty-six weeks, with progressive improvement and presumptive cure. Bacteriologic studies testing resistance to isoniazid and cycloserine in these cases are in progress and will be reported elsewhere.

As noted above, except for a single neurologic reaction among the 30 patients

in this study treated with 0.5 gm. of cycloserine, there was no other evidence of toxicity.

In addition, a group of 30 patients with pulmonary tuberculosis was treated on this service. The patients received a daily dose of 0.5 gm. of cycloserine as the only therapy for six months. These patients have shown no evidence of toxicity. The U. S. Public Health service report (4) gives data on 13 patients who received 0.25 gm. twice daily without toxic symptoms. Similar results have been reported by one of the Veterans Administration hospitals (15) on 15 patients, and by Robitzek (16*a*) on 29 patients. It is evident from these four studies, including more than 100 patients, that cycloserine in doses of 0.25 gm. given twice a day, alone or in combination with isoniazid, is as safe to use in the treatment of pulmonary tuberculosis as any currently used antimicrobial regimen.

Summary

A group of 43 previously untreated patients with pulmonary tuberculosis has been treated with cycloserine and isoniazid. An additional 14 patients were treated with cycloserine and streptomycin.

The administration of cycloserine in full daily doses of 1.0 gm. with 300 mg. of isoniazid per day, or 1.0 gm. of streptomycin twice a week, was not associated with any additive toxic action.

The administration of 1.0 gm. of cycloserine with 300 mg. of isoniazid per day elicited prompt and marked antituberculous activity, as shown by clinical improvement, rate of weight gain, roentgenographic clearing, and reversal of infectiousness.

Reduction of the daily dose of cycloserine to 0.5 gm. and of isoniazid to 4.0 mg. per kg. appears not to have altered the therapeutic efficacy of the combination. Clinical response and roentgenographic clearing were prompt and marked in most cases, together with evidence of rapid reversal of infectiousness.

Reduction of the daily dose of cycloserine lessened the incidence of side reactions. There was only one questionable reaction among the 63 subjects so far treated with 0.25 gm. of cycloserine used twice daily, either alone or in combination with isoniazid.

One gram of cycloserine per day combined with 1.0 gm. of streptomycin twice a week gave results that were only slightly less prompt and smaller in degree than they were with the isoniazid-cycloserine combinations, but similar to those from the usual isoniazid-PAS regimen.

Clinical resistance to isoniazid-cycloserine therapy has not developed, as judged by progressive roentgenographic response. Sputum conversion has been so rapid as to preclude testing for resistant bacilli in most acute cases after twenty-four weeks of therapy.

The use of smaller doses of cycloserine than heretofore employed, in combination with isoniazid, has resulted in a nontoxic, highly effective regimen for the treatment of pulmonary tuberculosis. Evidence has been presented that this combination has proved to be superior to other therapy in speed and degree of clinical response.

Summario

La Cicloserina Combinada con Otros Agentes Antituberculosos en el Tratamiento de la Tuberculosis Pulmonar

El principio de la terapéutica combinada ha sido aplicado a la tuberculosis mediante el uso de cicloserina e isoniacida o estreptomicina. De los datos obtenidos hasta ahora cabe sacar las siguientes conclusiones:

La cicloserina a plenas dosis diarias de 1.0 gm. es compatible con 300 mg. de isoniacida al día o 1.0 de estreptomicina dos veces semanales, sin observarse ningún efecto tóxico aditivo.

La combinación de 1.0 gm. de cicloserina con 300 mg. de isoniacida al día aportó pruebas de rápida y destacada actividad antituberculosa, según demostraron la mejoría clínica, el índice de aumento de peso, el despejo radiográfico y el viraje de la infecciosidad.

La reducción de la dosis diaria de cicloserina a 0.5 gm. y de isoniacida a 4.0 mg. por kg. de peso no parece haber alterado la eficacia terapéutica de la combinación. La respuesta clínica y el despejo radiográfico fueron rápidos y notables en la mayoría de los casos, con signos de rápida inversión de la infecciosidad.

La reducción de la dosis diaria de cicloserina minoró la incidencia de reacciones colaterales. No hubo más que una reacción dudosa entre los 63 sujetos tratados hasta ahora con 0.25 gm. de cicloserina usada dos veces al día, ya sola o combinada con isoniacida.

Un gramo de cicloserina al día, combinado con 1.0 gm. de estreptomicina dos veces semanales, dió resultados apenas menos rápidos e intensos que los conseguidos con las combinaciones de cicloserina-isoniacida, pero semejantes a los del régimen habitual de isoniacida-PAS.

A juzgar por la progresiva respuesta radiográfica, no se ha producido resistencia clínica a la cicloserino-isoniacido-terapia. El viraje de la infecciosidad ha sido tan rápido que vedó la comprobación en busca de bacilos resistentes en la mayoría de los casos agudos al cabo de veinticuatro semanas de terapéutica.

El empleo de dosis de cicloserina más pequeñas que las usadas hasta ahora, en combinación con la isoniacida, ha dado por resultado un régimen atóxico y sumamente eficaz para el tratamiento de la tuberculosis pulmonar. Se presentan aquí pruebas de que esta combinación se ha mostrado superior a otra terapéutica en la celeridad y la intensidad de la respuesta clínica.

Resume

Cyclosérine associée à d'autres agents antituberculeux dans le traitement de la tuberculose pulmonaire

Le principe du traitement combiné a été appliqué au traitement de la tuberculose à l'aide de la cyclosérine et de l'isoniazide ou de la streptomycine. Les données obtenues jusqu'à présent permettent les conclusions suivantes:

La cyclosérine à dose totale de 1 g par jour est compatible avec 300 mg d'isoniazide par jour ou 1 g de streptomycine deux fois par semaine. Aucune toxicité additionnelle n'a été observée.

L'association de 1 g de cyclosérine à 300 mg d'isoniazide par jour a fait preuve d'une activité antituberculeuse rapide et accentuée comme le démontre le gain pondéral, l'amélioration radiologique et la négativation de l'expectoration.

La réduction à 0.50 g de la dose quotidienne de cyclosérine et de celle de l'isoniazide à 4 mg/kg ne paraît pas avoir altéré l'efficacité thérapeutique de cette association médicamenteuse. La réponse clinique et l'amélioration radiologique se sont manifestées promptement et de façon marquée dans la plupart des cas, qui sont rapidement devenus abacillaires.

La réduction de la dose quotidienne de cyclosérine diminuait l'incidence des réactions secondaires. Une seule réaction douteuse a été observée parmi les 63 sujets ayant été traités jusqu'à présent avec 0.25 g de cyclosérine deux fois par jour administrée seule ou en association avec l'isoniazide.

Les résultats obtenus avec 1 g de cyclosérine par jour associé à 1 g de streptomycine deux fois par semaine ont été légèrement moins rapides à se manifester et moins marqués qu'avec l'association cyclosérine-isoniazide. Toutefois, ils étaient semblables à ceux obtenus avec le traitement usuel isoniazide-PAS.

La résistance clinique au traitement combiné cyclosérine-isoniazide ne s'est pas produite comme l'a prouvé l'amélioration radiologique progressive. La rapidité de la négativation du crachat a été telle, qu'au bout de 24 semaines de traitement les tests d'isolement des bacilles résistants ont été considérés inutiles dans la plupart des cas aigus.

L'emploi de doses de cyclosérine plus faibles que celles employées jusqu'à présent, en association avec l'isoniazide, constitue une thérapeutique non toxique et d'une grande efficacité pour le traitement de la tuberculose pulmonaire. Les auteurs présentent des données démontrant que cette association est supérieure aux autres médications en ce qui concerne sa rapidité d'action et le degré des résultats cliniques.

Acknowledgment

The writers are grateful to Michael G. Mulinos, Medical Director of Commercial Solvents Corporation, for supplies of cycloserine and for the valuable help and advice he has rendered throughout this investigation. They are also indebted to Mrs. Ann Griffiths, bacteriologist, Mr. A. Berelson, roentgenologist, and Dr. Paul Auspitz, visiting physician of the Metropolitan Hospital, for their help in pursuit of this project.

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FIGURES 1–6 will follow

A B C

FIG. 1

GROUP IA

GROUP *IA*

Dosage regimen: Cycloserine, 1.0 gm., and isoniazid, 300 mg. per day orally (figures 1 and 2).

FIG. 1. M. B., a 23-year-old Puerto Rican male, was admitted to the hospital on April 1, 1955, with a low-grade fever. He had an acute recent tuberculous pneumonic lesion in the right upper lobe with cavitation and sputum positive for tubercle bacilli. He was started on 250 mg. of cycloserine four times a day and 150 mg. of isoniazid twice daily on April 14, 1955.

Prompt symptomatic improvement occurred. The patient gained weight rapidly (25 pounds to date). Sputum became negative for tubercle bacilli on smear and culture in the twelfth week of therapy and has continued to be noninfectious.

The patient was transferred to the outpatient department on September 6, 1955. He has been continued on the above therapy for a total of one year. The patient has had no evidence of side reactions, and repeated blood, liver, and kidney function studies have remained unchanged.

A: Chest roentgenogram taken on admission and at the start of isoniazid-cycloserine therapy, showing caseous pneumonic tuberculous lesion involving the upper half of the right lung with a cavity between the first and second right anterior ribs.

B: Chest roentgenogram showing that within less than two months there has been a great deal of clearing of the caseous tuberculous disease in the right lung. The cavity could not be outlined.

C: Roentgenogram obtained after ten months of treatment showing that there has been complete clearing of all pulmonary abnormalities.

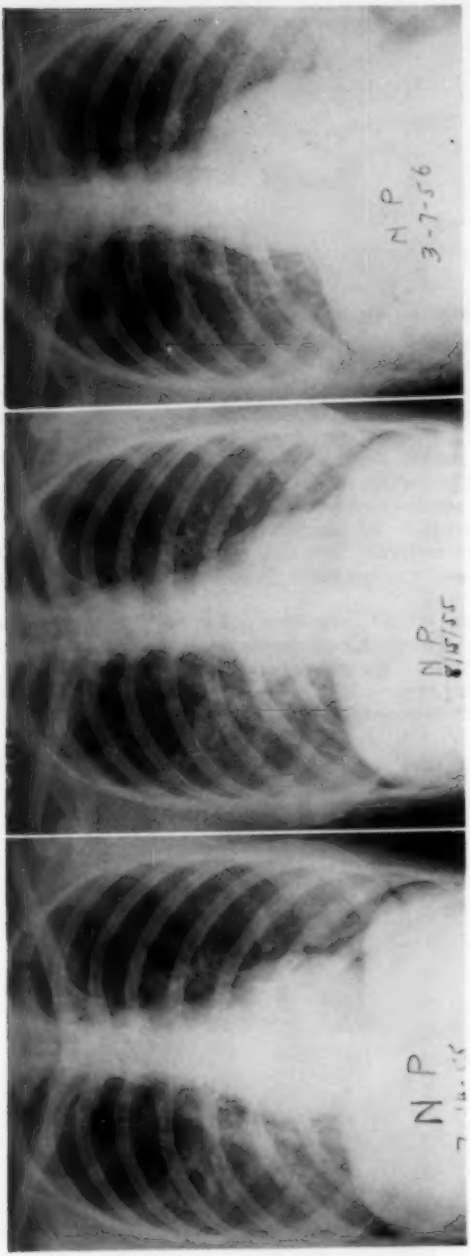


FIG. 2

GROUP 1A (*Continued*)

FIG. 2. N. P., a 23-year-old Puerto Rican female, was admitted to the hospital in July, 1955, with a high fever and hemoptysis. She had a caseous pneumonic tuberculous lesion in the right lung, with cavitation and sputum positive for tubercle bacilli. She was started on 250 mg. of cycloserine four times a day and 150 mg. of isoniazid twice daily on July 19, 1955.

There was a quick response, as seen by rapid defervescence and disappearance of toxemia. Sputum became negative for tubercle bacilli on smear and culture by the eighth week and has continued to be noninfectious. The patient has gained 36 pounds in thirty-six weeks. The antimicrobial therapy was well tolerated, there being no side reactions. Repeated blood studies and kidney and liver function tests have remained within pretreatment levels.

A: Chest roentgenogram obtained at the onset of therapy, showing caseous pneumonic tuberculous disease in the lower two-thirds of the right lung with a cavity in the right midlung field.

B: Chest roentgenogram showing that, within one month, there has been a good deal of clearing of the extensive abnormalities in the right lung. The cavity can no longer be outlined.

C: Chest roentgenogram obtained eight months after the onset of therapy, showing clear lungs.

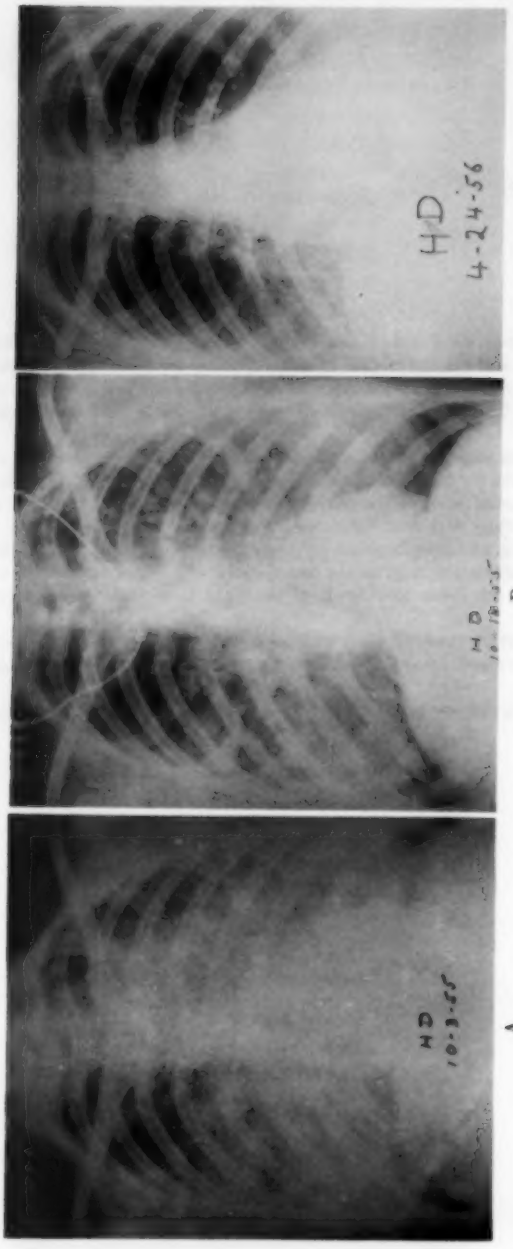


FIG. 3

GROUP IB

GROUP *IB*

Dosage regimen: Cycloserine, 0.5 gm., and isoniazid, 4.0 mg. per kg. per day, orally (figures 3, 4, and 5)

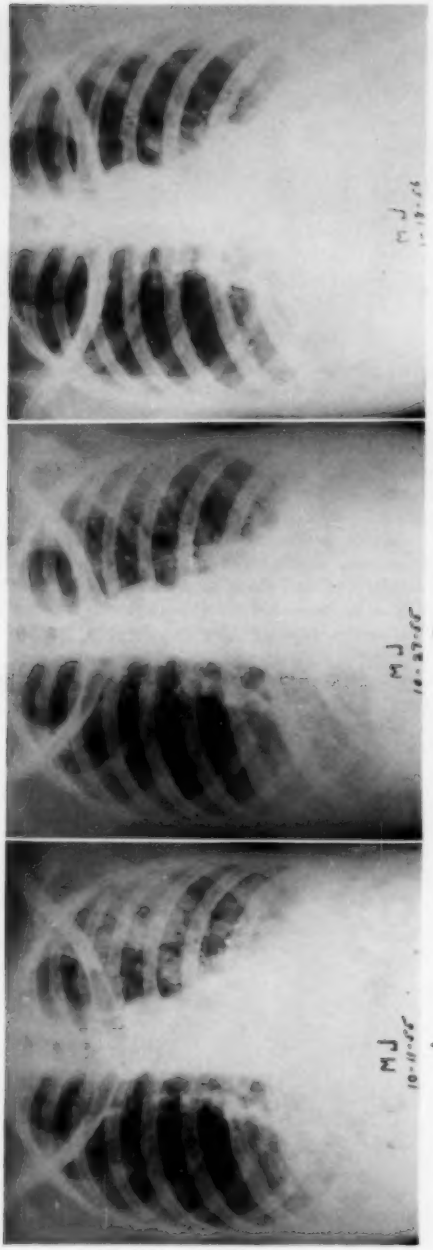
FIG. 3. H. D., an 18-year-old Puerto Rican female, was admitted to the hospital on October 1, 1955, with a temperature of 102°F. She had an acute recent bilateral pneumonic disease and sputum positive for tubercle bacilli. She was started on 250 mg. of cycloserine and 2.0 mg. per kg. of isoniazid twice daily.

The patient improved promptly. The temperature dropped to normal on the fourth day and has continued to be normal. Sputum became noninfectious on smear and culture after six weeks of therapy. After seven months of therapy she is ambulatory and has gained 36 pounds. Therapy was well tolerated, and there were no side reactions. Frequent blood studies and kidney and liver function tests did not change during the treatment.

A: Chest roentgenogram obtained at onset of therapy, showing a diffuse caseous pneumonia involving most of both lungs.

B: Chest roentgenogram obtained two weeks after onset of therapy already showing a marked clearing in both lungs.

C: Chest roentgenogram obtained after less than six months' treatment, showing complete clearing of the originally extensive bilateral pulmonary abnormalities.



A B C

FIG. 4

GROUP 1B *(Continued)*

FIG. 4. M. J., a 22-year-old Negro female, was admitted to the hospital on October 4, 1955, with an acute history of disease of two weeks' duration. She was highly toxic with a temperature of 104°F. and sputum positive for tubercle bacilli. She had left upper lobe cavitary tuberculous disease with dissemination to the lower lobe.

The patient was started on 250 mg. of cycloserine and 2.0 mg. per kg. of isoniazid twice daily on October 8, 1955. The fever dropped slowly because of a concomitant nontuberculous pelvic inflammatory disease. During seven months of therapy she gained 15 pounds. Sputum became negative for tubercle bacilli on smear and culture after the sixth week and has remained noninfectious. Therapy was well tolerated, with no side reactions. Repeated blood studies and kidney and liver function tests did not change during the twenty-nine weeks of treatment.

A: Chest roentgenogram obtained at onset of therapy, showing a left upper lobe caseous tuberculous disease with cavitation and a dissemination into the left lower lung field.

B: Chest roentgenogram obtained two weeks after 4*A* showing a great deal of clearing in the lungs. The left upper lobe cavity could no longer be clearly outlined.

C: Chest roentgenogram taken three months later, showing that within that time there has been almost complete clearing of the abnormalities in the patient's lungs; only minimal productive changes remain in the left upper lobe.

A B C

FIG. 5

GROUP 1B (*Continued*)

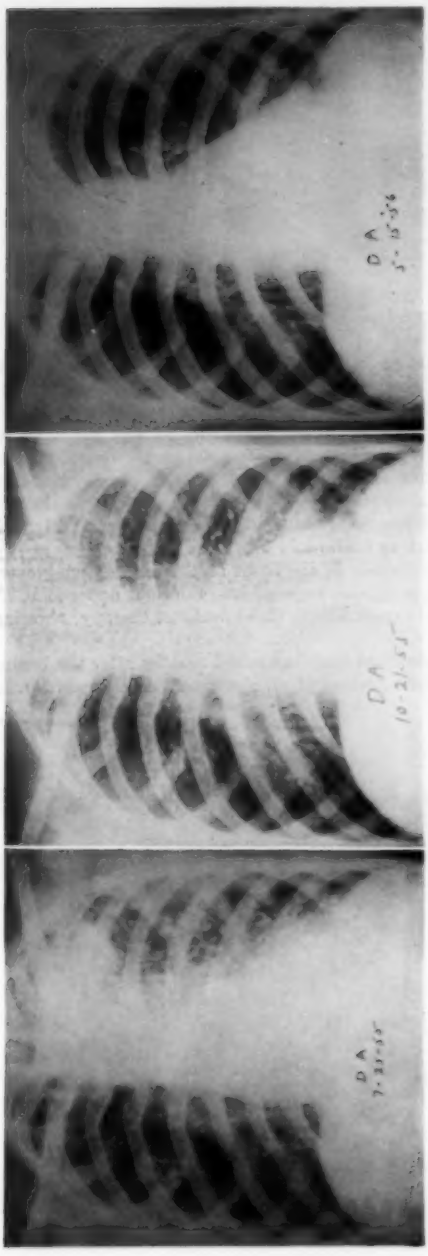
FIG. 5. F. W., an 18-year old Negro male, was admitted to the hospital in October, 1955 with a temperature of 102°F. and severe pain in the left chest. Sputum was positive for tubercle bacilli and a roentgenogram showed a caseous pneumonic process in the left lung.

The patient was placed on 250 mg. of cycloserine and 2.0 mg. per kg. of isoniazid twice daily on October 21, 1955. Temperature fell to normal on the fourth day and continued normal. He has gained 17 pounds in twenty weeks. Sputum became negative for tubercle bacilli on smear and culture after eight weeks of therapy and has remained noninfectious. There were no side reactions. Repeated blood studies and kidney and liver function tests did not show any changes during therapy.

A: Chest roentgenogram obtained at the onset of therapy, showing extensive caseous pneumonic disease of the left lung with a spread into the right upper lobe.

B: Chest roentgenogram obtained within three weeks of therapy, showing a great deal of clearing in both lungs.

C: Chest roentgenogram obtained less than seven months after the beginning of treatment, showing almost complete clearing of all abnormalities except for minimal residual productive changes.



A B C

FIG. 6

GROUP II

GROUP II

Dosage regimen: Cycloserine, 1.0 gm. per day, orally, and streptomycin, 1.0 gm. twice weekly, intramuscularly (figure 6)

FIG. 6. D. A., a 19-year-old Puerto Rican male, was admitted to the hospital on July 15, 1955, with a temperature of 100°F.

The patient had an acute recent caseous pneumonic lesion in the left upper lobe with dissemination to the lower and right upper lobes. Sputum was positive for tubercle bacilli. He was placed on 250 mg. of cycloserine four times a day and 1.0 gm. of streptomycin biweekly.

The fever fell on the third day and has remained normal. Sputum became negative for tubercle bacilli on smear and culture by the eighth week and has continued so. The patient has gained twenty pounds during the ten months of therapy. The therapy was well tolerated and there were no side reactions. Repeated blood studies and kidney and liver function tests did not show any changes during this study.

A: A chest roentgenogram obtained at the onset of therapy, showing an extensive caseous disease involving the left upper lobe with a spread into the left lower and right upper lobes.

B: Chest roentgenogram obtained after three months of therapy, showing a great deal of clearing in both lungs.

C: Chest roentgenogram obtained after ten months of therapy, showing that, within that time, there was almost complete clearing of the abnormalities in both lungs with only vaguely minimal productive changes in the left upper lobe.

STREPTOVARICIN[1]

I. Discovery and Biologic Activity

PAUL SIMINOFF, ROBERT M. SMITH, WALTER T. SOKOLSKI, AND G. M. SAVAGE

(Received for publication December 12, 1956)

INTRODUCTION

Streptovaricin,[2] a newly discovered antimicrobial drug, is of particular interest because of its high degree of activity against *Mycobacterium tuberculosis* both *in vitro* and *in vivo*. The antimicrobial is also active *in vitro* against a number of gram-positive, gram-negative, and saprophytic acid-fast bacteria.

Streptovaricin is produced under conditions of submerged fermentation by a hitherto undescribed actinomycete isolated from a soil sample collected in Dallas, Texas, which has been named *Streptomyces spectabilis*, n. sp. Papers concerning the taxonomy of *S. spectabilis* and the fermentation conditions under which streptovaricin is produced will be published elsewhere.

The present report describes briefly biologic assay procedures, paper chromatographic identification, and some *in vitro* characteristics of streptovaricin. Chemical properties and *in vivo* activity are presented in companion papers (1, 2).

MATERIALS AND METHODS

Assays: Microbiologic assays of streptovaricin were performed by an adaptation of the agar diffusion method of Loo and associates (3) or by a turbidimetric procedure, both techniques employing *Mycobacterium ranae* (Upjohn Culture Collection No. 161) as the test organism. A partially crystalline material containing a complex of the streptovaricin components was used as the standard. The plating medium for the agar diffusion method was brain-heart infusion (Difco®) to which had been added 1.5 per cent agar.

The liquid medium for the turbidimetric procedure was brain-heart infusion containing 1 per cent Tween® 80. Seeded tubes containing standard solutions and unknowns were incubated overnight on a reciprocal shaker at 37°C. Optical densities were read in a Lumetron® colorimeter at 650 mμ, and potencies of the unknowns were computed from a standard curve plotted as optical density *versus* log concentration.

Paper chromatography: Sheets of 6- by 22-inch Schleicher and Schuell #589 (Blue Ribbon special) paper were impregnated with 0.2 M phosphate buffer at pH 4.1 and air dried. The developing solvent system was prepared by mixing in a separatory funnel cyclohexane, chloroform, and distilled water in a volume ratio of 1:8:2, respectively, and allowing the aqueous and organic phases to separate. After solutions of streptovaricin were spotted and dried, the strips were equilibrated for two hours in the atmosphere of both phases and developed for five hours in the organic phase. The strips were then dried in a forced-air oven at 37°C. and plated out on brain-heart-infusion agar seeded with *Mycobacterium ranae*.

In vitro antimicrobial activity: Partially crystalline, antimicrobially pure specimens of the complex and components were tested against a number of bacteria by twofold dilution in brain-heart infusion adjusted to pH 6.8 before sterilization. All tests were incubated at 37°C. except for *Pseudomonas aeruginosa* and *Bacillus subtilis*, which were incubated at

[1] From the Research Laboratories, The Upjohn Company, Kalamazoo, Michigan.

[2] The trade name of The Upjohn Company for the antimicrobial streptovaricin is Dalacin.

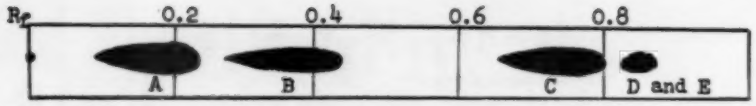


FIG. 1. Position of streptovaricin components in a paper chromatogram using strips buffered at pH 4.1 and a developing solvent of cyclohexane-chloroform-water in a volume ratio of 1:8:2. The bio-autographs are developed with *M. ranae*.

32°C. End points were read at the end of sixteen and forty hours. Tests against *M. tuberculosis* H37Rv and *M. tuberculosis* BCG were conducted in Kirchner broth containing 2 per cent (v/v) Dubos medium albumin (Difco). End points were determined at the end of seven days of incubation at 37°C. Antifungal activity was determined by an agar dilution method in a medium which had the following composition in grams per liter of distilled water: dextrose, 10; peptone, 5; yeast extract, 1; agar, 20; pH adjusted to 6.8.

RESULTS

Identity: In the paper chromatographic system described, streptovaricin is resolved into four detectable components. The activities appearing at Rf's[3] 0.13, 0.37, and 0.77 are single components which have been named streptovaricins *A*, *B*, and *C*, respectively. The fourth activity appearing at Rf 0.88 was shown by comparison with fractions obtained by physicochemical methods to be an unresolved mixture of two additional components, *D* and *E* (figure 1). The name streptovaricin will refer in this paper to the complex, while each component will be referred to with the appropriate letter.

In vitro antimicrobial activity: The antibacterial spectrum is shown in table 1. The complex and streptovaricins *A*, *B*, and *C* were active against a variety of gram-positive, gram-negative, and acid-fast microorganisms. Activity was especially pronounced against the human and attenuated BCG strains of *M. tuberculosis*. Component *A* appeared to be the most active of the family against *Streptococcus hemolyticus*, *Diplococcus pneumoniae*, and *Proteus vulgaris*.

The *D* and *E* components did not inhibit any of the gram-negative bacteria at 100 γ per ml. and showed a relatively low order of activity against gram-positive bacteria and the mycobacteria. They will not be considered in further detail in this paper.

Streptovaricin inhibited the following fungi at 100 γ per ml.: *Nocardia asteroides*, *Blastomyces dermatitidis*, *Hormodendrum compactum*, *Phialophora verrucosa*, *Cryptococcus neoformans*, *Sporotrichum schenckii*, *Monosporium apiospermum*, *Trichophyton rubrum*, *Microsporum audouini*, *Microsporum canis*, and *Trichophyton violaceum*. No inhibition was observed against the following fungi at 1,000 γ per ml.: *Coccidioides immitis*, *Geotrichum sp.*, *Trichophyton interdigitale*, *Candida albicans* Abbott, and *Candida albicans* Upjohn.

In table 1 it may be seen that displacement of minimal inhibitory concentration to higher concentration of the antimicrobial takes place if incubation of the antibacterial test is prolonged to forty hours. This end-point shift suggested that

[3] Rf (ratio of fronts) is a term denoting the ratio of distance of travel of the material under consideration and the distance of travel of the developing solvent.

TABLE 1

ANTIBACTERIAL SPECTRUM OF STREPTOVARICIN

	Minimal Inhibitory Concentration (γ/ml.)							
	Complex		Component *A*		Component *B*		Component *C*	
	16 Hours	40 Hours	16 Hours	40 Hours	16 Hours	40 Hours	16 Hours	40 Hours
Micrococcus pyogenes var. *aureus* 284	0.39	1.56	0.39	0.78	0.39	0.78	0.78	1.56
Micrococcus albus 151	0.39	1.56	0.20	0.78	0.20	0.78	0.78	3.12
Streptococcus hemolyticus C203	25.00	>100	3.12	50.00	25.00	>100	25.00	>100
Streptococcus viridans 25-11	3.12	25.00	3.12	25.00	3.12	25.00	12.50	>25
Bacillus subtilis III	0.39	1.56	0.39	3.12	0.20	1.56	0.78	3.12
Escherichia coli 26	25.00	100.00	25.00	50.00	12.50	100.00	25.00	100.00
Proteus vulgaris 8427	100.00	>100	25.00	100.00	100.00	>100	>100	>100
Klebsiella pneumoniae AD	3.12	12.50	3.12	6.25	3.12	12.50	6.25	12.50
Diplococcus pneumoniae FSI	6.25	50.00	1.56	6.25	12.50	>100	100.00	>100
Pseudomonas aeruginosa 9027	12.50	50.00	12.50	50.00	12.50	50.00	25.00	100.00
Salmonella paratyphi B#4	>100	>100	>100	>100	>100	>100	>100	>100
Salmonella typhosa TG3	25.00	100.00	12.50	100.00	25.00	100.00	25.00	100.00
Pasteurella multocida 449	1.56	25.00	0.78	1.56	1.56	6.25	1.56	3.12
Mycobacterium ranae	0.78	6.25	0.39	1.56	0.78	3.12		
	Seven Days		Seven Days		Seven Days		Seven Days	
Mycobacterium tuberculosis H37Rv	0.16		0.08		0.26		0.31	
Mycobacterium tuberculosis BCG	0.16		0.16		0.31		0.31	

TABLE 2
STABILITY OF STREPTOVARICIN AT 37°C.

Preparation	Per Cent of Original Activity											
	Water				Buffer				Brain-Heart Infusion			
	Hours											
	0	16	40	64	0	16	40	64	0	16	40	64
Complex	100	63	14	18	100	25	4	5	100	39	11	9
Component *A*	100	38	15	7	100	29	3	0	100	39	9	3
Component *B*	100	40	10	10	100	24	<2	0	100	33	10	4
Component *C*	100	48	23	18	100	38	18	13	100	55	10	17

the antimicrobial might have lost activity during the test or that resistance development was manifested by the microorganisms.

Stability: The stability of streptovaricin in solution was measured in the following way: The complex and components *A*, *B*, and *C* were diluted aseptically in distilled water, M/10 phosphate buffer at pH 6.8, and brain-heart-infusion broth at pH 6.8 to concentrations of 40 to 70 γ per ml. equivalent to the standard. Samples taken at intervals from zero time through sixty-four hours' incubation at 37°C. were assayed for residual activity.

The data in table 2 indicate that streptovaricin is quite unstable in solution at 37°C., the greatest loss of potency occurring in the pH 6.8 buffer. Most of the activity loss occurred during the first forty hours. Streptovaricins *A* and *B* appeared to be more highly labile than the complex or component *C*. In fact, the results suggest that the greater stability of the complex over the *A* and *B* components reflected, at least in part, the presence of *C* in its composition.

Further studies have shown that the instability of streptovaricin is of the same magnitude whether incubated in the presence or absence of bacteria. Thus, the activity loss observed in media is not due to microbial attack but to the action of physical and chemical agencies in the environment.

Resistance development: Twofold dilutions were prepared and inoculated with two gram-positive bacteria, *M. pyogenes* var. *aureus* and *S. hemolyticus*, and two gram-negative bacteria, *E. coli* and *Ps. aeruginosa* (first inoculation). End points were determined at sixteen and forty hours. Forty-hour growth in tubes of highest antimicrobial concentrations equal to growth in the control tube was reinoculated at 1:40,000 dilution into a fresh series of antimicrobial concentrations in broth; tubes containing partial growth at highest antimicrobial concentrations were inoculated at 1:200 dilution into the fresh dilution series (second inoculation). End points were again read at the end of the sixteen- and forty-hour growth period.

The results obtained were somewhat variable from experiment to experiment. Lack of uniformity in the population of the inocula is probably accountable. However, the data clearly indicate that, in a representative trial of the antimicrobial as shown in table 3, increased resistance does occur during the *in vitro* test. Two patterns of resistance development were manifested: (*1*) a slow development

TABLE 3
INSTANCES OF RESISTANCE DEVELOPMENT DURING BROTH DILUTION TEST

Test Organism	Minimal Inhibitory Concentration (γ/ml.)			
	First Inoculation		Second Inoculation	
	16 Hours	40 Hours	16 Hours	40 Hours
Complex				
M. pyogenes var. *aureus*	0.39	1.56	0.78	3.12
S. hemolyticus	25.00	100.00	100.00	200.00
E. coli	12.50	25.00	100.00	>400
Ps. aeruginosa	12.50	50.00	50.00	>400
Streptovaricin *A*				
M. pyogenes var. *aureus*	0.39	1.56	>100	>100
S. hemolyticus	6.25	50.00	12.5	50.00
E. coli	12.50	25.00	12.5	25.00
Ps. aeruginosa	12.50	50.00	12.5	>400
Streptovaricin *B*				
M. pyogenes var. *aureus*	0.39	0.78	1.56	6.25
S. hemolyticus	25.00	200.00	100.00	100.00
E. coli	6.25	25.00	12.50	50.00
Ps. aeruginosa	12.50	50.00	12.50	400.00
Streptovaricin *C*				
M. pyogenes var. *aureus*	0.78	1.56	1.56	6.25
S. hemolyticus	25.00	50.00	50.00	100.00
E. coli	25.00	50.00	100.00	>400
Ps. aeruginosa	12.50	100.00	25.00	200.00

which occurred during prolongation of the incubation period to forty hours, or (*2*) a rapid resistance development pattern in certain bacteria. In the first pattern, the nominal sixteen-hour end point of the second inoculation series simply reproduced the forty-hour end point, within experimental error, of the first inoculation group. A new end-point shift to higher antimicrobial concentrations occurred upon continuance of incubation to forty hours. Examples of this type were the response of *Ps. aeruginosa* to the complex and of *M. pyogenes* var. *aureus* to streptovaricin *B*. In the second pattern, the bacteria, upon reinoculation, appeared resistant to much more of the antimicrobial than was indicated by the forty-hour end point of the first inoculation series, e.g., the response of *E. coli* to the complex and of *M. pyogenes* var. *aureus* to component *A*.

In certain instances, end-point changes were not accompanied by significantly increased resistance to the antimicrobial. For example, the susceptibility of *M. pyogenes* var. *aureus* to the complex, *S. hemolyticus* to the *A* component, or

TABLE 4
CROSS-RESISTANCE OF BACTERIA TO THE COMPLEX AND COMPONENTS *A* AND *B*

Test Bacteria*	Minimal Inhibitory Concentration (γ/ml.)		
	Complex	Streptovaricin *A*	Streptovaricin *B*
E coli Complex-*R*	>400	>400	>400
Ps. aeruginosa Complex-*R*	>400	>400	>400
Ps. aeruginosa A-R	400.00	>400	400.00
Ps. aeruginosa B-R	400.00	>400	400.00
M. pyogenes var. *aureus A-R*	200.00	>400	>400
M. pyogenes var. *aureus B-R*	12.50	3.12	6.25

* Complex-*R*—Made resistant to the complex. *A-R*—Made resistant to component *A*. *B-R*—Made resistant to component *B*.

TABLE 5
CROSS-RESISTANCE OF STREPTOVARICIN *C*-RESISTANT BACTERIA TO THE COMPLEX AND COMPONENTS *A* AND *B*

Test Bacteria	Minimal Inhibitory Concentration (γ/ml.)			
	Complex	Streptovaricin *A*	Streptovaricin *B*	Streptovaricin *C*
E. coli C-R	>400		100	>400
Ps. aeruginosa C-R	200	200	100	200

TABLE 6
CROSS-RESISTANCE OF STREPTOVARICIN WITH SEVERAL ANTIMICROBIALS

Test Bacteria	Remarks	Minimal Inhibitory Concentration
		γ/ml.
M. pyogenes var. *aureus* UC-76	Susceptible strain	0.78
M. pyogenes var. *aureus* UC-488	Resistant to penicillin, chlortetracycline, oxytetracycline, chloramphenicol, and tetracycline	0.78
M. pyogenes var. *aureus* UC-391	Resistant to penicillin, chlortetracycline, oxytetracycline, tetracycline, and streptomycin	0.39
M. pyogenes var. *aureus* UC-317	Resistant to penicillin, chlortetracycline, oxytetracycline, streptomycin, and erythromycin	0.78
M. tuberculosis H37Rv	Resistant to streptomycin at 100 γ per ml.	0.10

E. coli to the *B* component were not appreciably different upon reinoculation from those obtained in the first inoculation group.

That the apparent increased resistance to the antimicrobials manifested was a true resistance development is shown in tables 4 and 5. Upon becoming resistant to the complex or one of the components, the microorganisms withstood com-

parable concentrations of the other components. This has been shown in one experiment with the complex and streptovaricins *A* and *B*. In another trial with streptovaricin *C*, two organisms which had become highly resistant to this component were also able to proliferate in relatively high concentrations of the complex and components *A* and *B* (tables 4 and 5).

Discussion

On the basis of the *in vitro* data, streptovaricin is active against a wide variety of bacteria. Its activity against the tubercle bacilli is particularly striking. Under the same conditions of test, streptovaricin is about ten times as active as streptomycin, about one hundred times as active as para-aminosalicylic acid, and about half as active as isoniazid. Furthermore, streptovaricin does not give rise to resistance to streptomycin or to any of the commonly employed antimicrobial drugs (table 6).

The *in vitro* antibacterial data obtained in broth are complicated by the instability of the antimicrobial under conditions of test. Apparently, when substantial end-point shifts occurred when incubation was continued beyond sixteen hours, some microbial resistance appeared. Whether such resistance is likely to occur so quickly under conditions of good antimicrobial stability cannot be answered at this time. It is probable that variability in the size of the populations inoculated also tended to influence the results. A more definitive analysis of the *in vitro* data must await determination of the rate of resistance emergence under conditions of a constant level of the antimicrobial and examination of the initial bacterial inocula for clones resistant to the antimicrobial.

Summary

A new antimicrobial complex, streptovaricin, is produced by *Streptomyces spectabilis*, n. sp.

The complex consists of at least five microbiologically active, closely related components which have been named streptovaricins *A*, *B*, *C*, *D*, and *E*, respectively. These components may be differentiated by paper chromatography.

The complex and its components are active *in vitro* primarily against *M. tuberculosis* and gram-positive bacteria, although several gram-negative bacteria are susceptible.

Streptovaricin does not give rise to cross resistance to streptomycin, or to any other commonly used antimicrobial drug.

Susceptible bacteria show increased resistance to streptovaricin after exposure. Some of the factors concerned with this increased resistance have been studied.

Sumario

Estreptovaricina. I. Descubrimiento y Actividad Biológica

Un nuevo complejo antimicrobiano, la estreptovaricina, es producido por el *Streptomyces spectabilis*, n. sp.

El complejo consta a lo menos de cinco componentes íntimamente afines y microbiológicamente activos que han sido denominados estreptovaricinas *A*, *B*, *C*, *D* y *E*, respectivamente. Estos componentes pueden identificarse con la cromatografía en papel.

El complejo y sus componentes son activos *in vitro* primariamente contra el *M. tuberculosis* y las bacterias gram-positivas, aunque varias bacterias gram-negativas son también susceptibles.

La estreptovaricina no muestra resistencia cruzada con la estreptomicina ni con ninguna otra droga antimicrobiana usada comúnmente.

Las bacterias susceptibles despliegan mayor resistencia a la estreptovaricina después de la exposición a ésta. Se estudian algunos de los factores que intervienen en este aumento de la resistencia.

Resume

Streptovaricine: 1. Découverte et Activité biologique

Un complexe antimicrobien nouveau, la streptovaricine, est produit par *Streptomyces spectabilis*, n. sp.

Ce complexe comprend au moins cinq composants actifs du point de vue microbiologique et étroitement apparentés. Ils ont été respectivement dénommés: streptovaricine *A. B. C. D.* et *E.* Ces trois composants peuvent être différenciés par la chromatographie sur papier.

Ce complexe et ses composants sont actifs *in vitro* surtout contre le *M. tuberculosis* et les bactéries gram-positives, bien que plusieurs bactéries gram-négatives soient également sensibles à leur action.

La streptovaricine ne donne pas de résistance croisée avec la streptomycine ou avec d'autres agents antibactériens d'usage courant.

Les bactéries sensibles présentent un accroissement de résistance à la streptovaricine après y avoir été exposées. Certains des facteurs intéressés dans cet accroissement de résistance ont été étudiés.

Acknowledgments

The writers gratefully acknowledge the supplies of streptovaricin components which were supplied by Drs. Ross Herr, George B. Whitfield, and Gerald A. Boyack. They also acknowledge the valuable technical assistance rendered by the Misses Alma Dietz, Nancy J. Eilers, and Lorna Gregory, and by Messrs. Ernest C. Perin, Richard Keene, Milton R. Burch, E. D. Rogers, and A. R. Barbiers.

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STREPTOVARICIN[1]

II. Isolation and Properties

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(Received for publication December 12, 1956)

INTRODUCTION

Streptovaricin[2] is a new, partially crystalline complex of antimicrobials produced by a previously unidentified actinomycete which has been named *Streptomyces spectabilis*. This complex is very active in protecting mice experimentally infected with *Mycobacterium tuberculosis* H37Rv. The *in vitro* and *in vivo* characteristics are given in accompanying papers (1, 2).

ISOLATION AND PURIFICATION

Streptovaricin is isolated from the culture filtrate by extraction with ethyl acetate at harvest pH. The extract is concentrated to a small volume and precipitated by addition to four volumes of technical hexane.[3] The precipitate is dissolved in methylene chloride and the

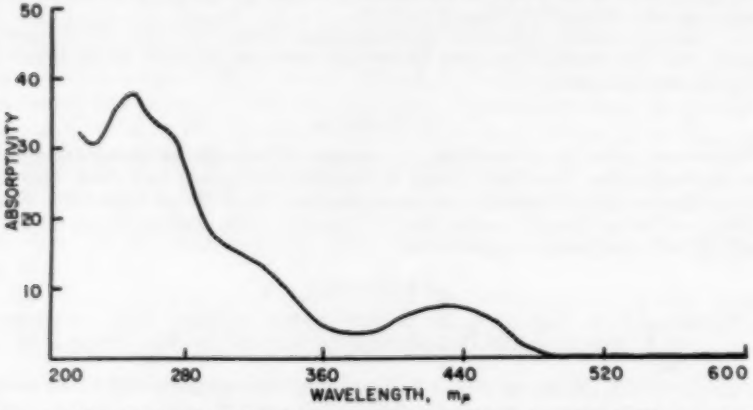

FIG. 1. Ultraviolet and visible absorption spectrum of streptovaricin in 95 per cent ethanol.

extract is precipitated with five volumes of hexane. The partially crystalline precipitate is washed with hexane and dried *in vacuo*. Reprecipitation causes no increase in potency. This complex has been named streptovaricin. It is composed of 60 per cent antimicrobials, 40 per cent inactive material. The inactive material resembles the complex and is separated only with difficulty. The complex is reproducible as indicated by paper chromatographic analysis.

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[1] From the Research Laboratories, The Upjohn Company, Kalamazoo, Michigan.

[2] The trade name of The Upjohn Company for the antimicrobial streptovaricin is Dalacin.

[3] Skellysolve B, Skelly Oil Co., Kansas City, Missouri.

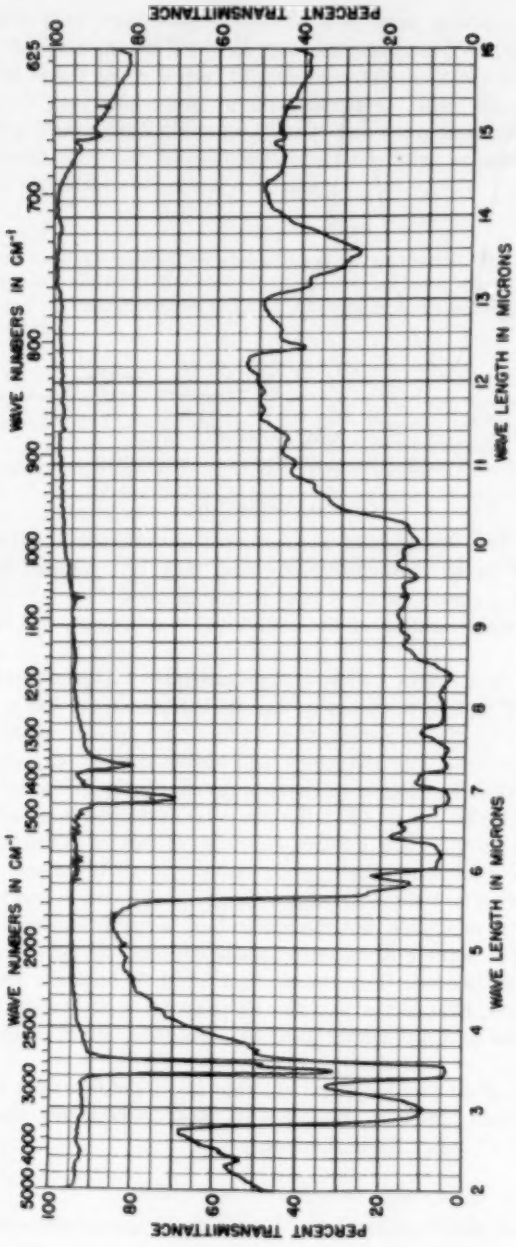

FIG. 2. Infrared absorption spectrum—streptovaricin (mineral oil mull).

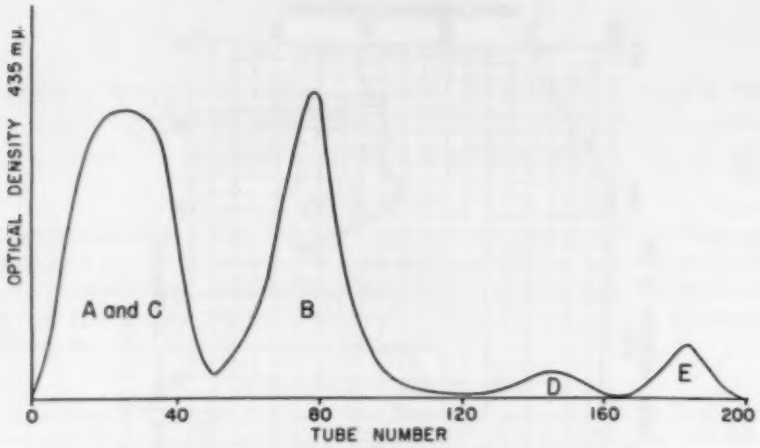

FIG. 3. Countercurrent distribution—streptovaricin.

CHARACTERIZATION

Streptovaricin is a complex of at least five antimicrobials. These may be distinguished by paper chromatography and may be separated by partition chromatographic columns or by Craig countercurrent distributions (3). The isolation and characterization of the individual components will be published elsewhere.

Streptovaricin is a bright yellow-orange material. A typical lot is optically active, $[\alpha]_D^{24}$ +288° (*c*, 0.31 in absolute ethanol). Streptovaricin has a characteristic maximum in the visible region, this lot having a specific absorbance, *a*, of 7.7 at 435 millimicrons. The ultraviolet and visible absorption spectrum of the complex is reproduced in figure 1. There is no change in this spectrum in acid solution, but the maximum at 435 millimicrons is destroyed in alkaline solution, as is the biologic activity. The infrared spectrum of this lot is shown in figure 2.

Streptovaricin is labile to alkali, losing practically all of its activity in three days at pH 7.8. It is an indicator, being yellow in acid solution and red-amber in alkali. The color is reversible, but the loss in bioactivity is irreversible.

Streptovaricin is soluble in dimethylformamide and 95 per cent ethanol in excess of 500 mg. per ml. It is soluble in methanol, butanol, lower ketones, methylene chloride, chloroform, and the lower acetates. It is slightly soluble in water (0.7 mg. per ml.) and nearly insoluble in hexane, ether, carbon tetrachloride, and benzene.

Streptovaricin gives positive ferric chloride and iodoform tests but negative Benedict, ninhydrin, Molisch, Zimmerman, and Liebermann tests. This lot melts at 144–147°C. (dec., Kofler hotstage).

SEPARATION OF COMPONENTS

Countercurrent distributions have been run on streptovaricin. Using the solvent system consisting of equal parts of water, acetone, and ethyl acetate,

streptovaricin gave one peak, $k = 3.81$. The band is wider than theoretical, indicating the presence of at least two components. The solvent system (4) consisting of equal parts water, 95 per cent ethanol, cyclohexane, and ethyl acetate, gives good resolution in 200 transfers. Three components separate cleanly and a fourth peak contains at least two components since it is broader than theoretical (figure 3). These components are being isolated in large enough amounts for characterization and evaluation.

Acknowledgments

The writers wish to express their appreciation to the various members of The Upjohn Company who contributed to this work, and in particular to Mr. Glen Crum and associates for the large-scale preparations and to Dr. J. L. Johnson and associates for the ultraviolet and infrared studies.

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STREPTOVARICIN[1]

III. *In Vivo* Studies in the Tuberculous Mouse

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Introduction

Streptovaricin[2] has been reported by Siminoff and associates (1) to be active *in vitro* against both gram-positive and gram-negative organisms. In addition, the antimicrobial exhibits exceptional *in vitro* activity against human and bovine strains of tubercle bacilli.

This paper is concerned primarily with *in vivo* studies with streptovaricin in the tuberculous mouse. The terminology suggested by Siminoff and associates (1) in referring to the streptovaricin components will be used in this study, i.e., streptovaricin will refer to the antimicrobial complex, and the individual components will be referred to by the appropriate letter.

Methods and Materials

Mycobacterium tuberculosis var. *hominis* (H37Rv) and *Mycobacterium tuberculosis* var. *bovis* (Vallée) were maintained at 4°C. on Löwenstein agar slants. Virulence was maintained by mouse passage every six months. A four-day-old culture, transferred through three passages in Kirchner medium, was used as the inoculum for all *in vivo* experiments. The turbidity was adjusted to 68 to 70 per cent transmission at 660 mμ in a Beckman Model B spectrophotometer. Using these conditions, the ST_{50} of infected untreated mice was from 16 to 19 days.

Carworth Farms mice (CF-1 male) in a weight range of 16 to 18 gm. were used in the infected animal studies. The animals were injected in the tail vein with 0.5 ml. of culture, and treatment, when indicated, was initiated within three hours. Ten mice were used on each drug concentration. Unless indicated, all drugs were injected in 2.5 per cent gelatin in physiologic saline in a volume of 0.5 ml. by oral intubation or 0.25 ml. if administered by the subcutaneous route. Treatment was continued for 10 days, and the experiment was terminated after 28 days. The results were calculated by the method of Litchfield (2) and expressed as ΔST_{50} (increase in the mean survival time as compared with the infected untreated controls). The term minimal effective dose was used when drug comparisons were made, and this term designates a positive ΔST_{50} of 4 days. A statistical analysis of data obtained in this test system has revealed that the standard error of a ΔST_{50} determination is 2.0 days.

Observations

The exceptional *in vitro* activity of streptovaricin against mycobacteria prompted examination of this material in more detail in the tuberculous mouse. Initial six-day toxicity determinations revealed that mice could tolerate daily treatment, without appreciable weight loss, of 1,000 mg. per kg. when the drug was administered by oral intubation, or 800 mg. per kg. by the subcutaneous

[1] From the Research Laboratories, The Upjohn Company, Kalamazoo, Michigan.

[2] The trade name of The Upjohn Company for the antimicrobial streptovaricin is Dalacin.

TABLE 1

THE EFFECTIVENESS OF STREPTOVARICIN IN MICE INFECTED WITH MYCOBACTERIUM TUBERCULOSIS VAR. HOMINIS (H37Rv)

Streptovaricin	ΔST_{50} Days	
	Subcutaneously	Oral Intubation
mg/kg		
200	100% S*	100% S*
100	50% S*	70% S*
50	4.5	5.5
40	3.5	4.0
30	2.5	0.5
20	0	0

* Survivors at the end of the 28-day test period.

route. Preliminary *in vivo* experiments revealed that streptovaricin afforded 100 per cent protection to mice infected with *Mycobacterium tuberculosis* var. *hominis* (H37Rv) at concentrations of 200 mg. per kg. Subsequent determinations at lower drug concentrations (table 1) demonstrated that 40 mg. per kg., when administered either orally or by the subcutaneous route, afforded a significant degree of protection, ΔST_{50} of +4.0 days, in the *in vivo* system under study. This dosage concentration was selected as the minimal effective dose for streptovaricin and was used in subsequent comparative studies.

The antimicrobial was tested against a highly virulent strain of *Mycobacterium tuberculosis* var. *bovis* (Vallée) and found to be equally as effective (table 2) as it was against tubercle bacilli of human origin. In addition, it was observed that different research lots of streptovaricin could be obtained which exhibited the same relative protective activities in the tuberculous mouse, thus demonstrating that the streptovaricin complex could be consistently reproduced (table 3). These data, as do all others in the paper, represent a typical experiment. In all cases several determinations on each preparation have been made.

Comparative in vivo studies with streptovaricin A, B, and C: Chemical fractiona-

TABLE 2

STREPTOVARICIN EFFICACY IN MICE INFECTED WITH MYCOBACTERIUM TUBERCULOSIS VAR. BOVIS (VALLÉE)

Preparation	Drug Concentration	ΔST_{50} Days	
		Subcutaneously	Oral Intubation
	mg/kg		
Streptovaricin Lot #1	100	30% S*	60% S*
Streptovaricin Lot #1	50	9.0	11.5
Streptovaricin Lot #1	25	0.5	1.0
Streptovaricin Lot #2	100	80% S*	80% S*
Streptovaricin Lot #2	50	13.0	12.5
Streptovaricin Lot #2	25	3.0	3.0

* Survivors at the end of the 28-day test period.

TABLE 3

A COMPARISON OF THE IN VIVO ACTIVITY OF VARIOUS STREPTOVARICIN PREPARATIONS IN THE TUBERCULOUS MOUSE

Preparation	Drug Concentration	ΔST_m Days	
		Subcutaneously	Oral Intubation
	mg/kg		
Lot #1	50	4.6	5.6
Lot #2	50	6.5	9.1
Lot #3	50	5.2	6.5
Lot #4	50	2.0	1.5
Lot #5	50	5.0	5.5
Lot #6	50	3.5	6.5

tion of the streptovaricin complex by Whitfield and co-workers (3) resulted in isolation and purification of several of the components. The chemical studies have indicated a very close similarity between components as have the *in vitro* data reported by Siminoff and associates (1). It was interesting to note, therefore, that differences in the *in vivo* efficacy of the components could be determined in the tuberculous mouse (table 4). Streptovaricin *A* is not effective when administered by either the oral or subcutaneous route at concentrations as high as 400 mg. per kg., whereas streptovaricin *C* is more active than the streptovaricin complex. The results with streptovaricin *B*, indicating less activity than the complex, are equivocal since maximal purity of the preparation has not been attained.

In vivo studies with bacilli from streptovaricin-treated animals: In the early studies with streptovaricin, it was observed that mice infected with *M. tuberculosis* (H37Rv), followed by treatment for 10 days with a drug concentration affording 100 per cent survivors at the end of the 28-day test period, showed no gross evidence of tuberculosis. However, acid-fast stains made on tissue sections prepared from the lungs of these mice indicated the presence of numerous acid-fast organisms. Therefore, the remaining mice in the same experiment were allowed to live, without treatment, for a total period of 84 days. At the end of that time, the mice were sacrificed and the lungs were cultured for viable organisms after standard sodium hydroxide treatment. Viable acid-fast organisms were obtained which, when tested by standard broth dilution procedures, exhibited the same susceptibility to streptovaricin as the parent strain. Reinoculation of these cultures into mice demonstrated that the initial virulence of the culture had been maintained. In addition, mice infected with these cultures could be protected by the administration of streptovaricin. Similar experiments with isoniazid and streptomycin, in which drug concentrations affording 100 per cent survivors were used, have demonstrated that mice infected with virulent mycobacteria do not survive as long after therapy has been discontinued as do mice treated with streptovaricin, as described above.

Loss of virulence for mycobacteria made resistant to streptomycin: In vitro studies have demonstrated that *Mycobacterium tuberculosis* exhibits resistance to strep-

TABLE 4

A COMPARISON OF THE ANTITUBERCULOUS ACTIVITY OF SEVERAL STREPTOVARICIN COMPONENTS

Component	Drug Concentration	ΔST_{50} Days	
		Subcutaneously	Oral Intubation
	mg/kg		
Streptovaricin	50	4.5	5.5
Streptovaricin	30	2.5	0.5
Streptovaricin *A*	400	1.5	1.0
Streptovaricin *B*	100	6.5	2.5
Streptovaricin *B*	50	3.0	2.5
Streptovaricin *C*	50	9.0	9.0
Streptovaricin *C*	30	7.0	4.0

tovaricin at about the same rate as it does to isoniazid or streptomycin. Unlike the results reported for isoniazid (4), mycobacteria resistant to streptovaricin are attenuated for mice, although they remain catalase positive. In these mouse virulence studies, the inoculating procedure described in the Methods and Materials section was used and, over a period of 48 days, 100 per cent of the mice survived. It should be recalled that 50 per cent of the mice died 15 to 18 days post infection if the streptovaricin-susceptible mycobacterium was used. The loss of mouse virulence by streptovaricin-resistant mycobacteria does not appear to be due to serial transfer in Kirchner broth, since 19 serial transfers have been made with the susceptible strain of *M. tuberculosis* (H37Rv) without any significant change in the ST_{50} of infected, untreated mice.

DISCUSSION

The data described above illustrate very clearly the efficacy of streptovaricin and streptovaricin *B* and *C* in the tuberculous mouse.

In addition to its proved efficacy in the system under study, the observation that a short course of treatment with streptovaricin apparently inhibits the further outgrowth of tubercle bacilli in the tissues for an extended period of time merits further consideration. There are a number of preliminary experiments that should be considered here: A longer course of therapy has improved the effectiveness of the drug even in the fulminating type of infection obtained in the mouse. In the guinea pig, in which a 60-day period of treatment was used,[3] streptovaricin had been shown to cause regression of caseous lesions. In a chronic infection in mice, although viable cell counts (figure 1) of infected organs revealed that only a slow reduction in cells was obtained, the number of deaths obtained was significantly less than in the controls.

It should be recalled that McCune and associates (5) have reported that reduction of tubercle bacilli in lungs and spleen to less than a countable level through the use of combination drug therapy did not prevent microbial reappearance after therapy had been terminated. It is possible that, in the streptovaricin-

[3] Results to be published.

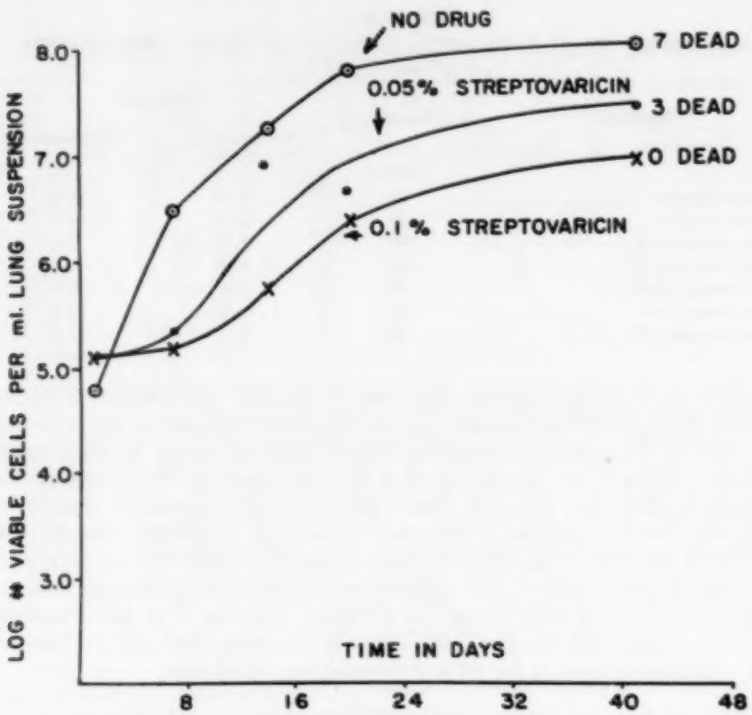

FIG. 1. The effect of streptovaricin on viable tubercle in the lungs of mice. Viable counts were done on Dubos-oleic-acid agar. The counts expressed in the figure are a result of the average count on three mice. In the figure, 0.05 and 0.1 per cent streptovaricin refer to amount of drug in the diet; 0.1 per cent equals about 200 mg. per kg.

treated animals, the bacilli found in the tissues, although apparently normal when cultured *in vitro*, may either have lost the power to multiply *in vivo* or are unable to produce the factors necessary for virulence. This thesis has, of course, an interesting implication in view of the exciting evidence presented by Segal and Bloch (6) demonstrating marked differences in the metabolism of mycobacteria grown *in vivo* when compared with their metabolism when grown *in vitro*.

SUMMARY

Streptovaricin has been shown to be highly effective in the tuberculous mouse. The antimicrobial consists of several components, two of which exhibit good *in vivo* activity. *M. tuberculosis* (H37Rv) does not develop resistance *in vivo* over an 84-day period, and *in vitro* resistant strains are attenuated for mice.

Sumario

Estreptovaricina. Estudios In Vivo en el Ratón Tuberculoso

Queda demostrado que la estreptovaricina es sumamente eficaz en el ratón tuberculoso. Este agente antimicrobiano consta de varios componentes, dos de los cuales manifiestan buena actividad *in vivo*. El *M. tuberculosis* (H37Rv) no despliega resistencia *in vivo* durante un período de 84 días, y cepas resistentes *in vitro* son atenuadas para los ratones.

Resume

Streptovaricine: Etude in vivo chez la souris tuberculeuse

La streptovaricine a fait preuve d'une haute efficacité chez la souris tuberculeuse. Cet antibactérien comporte plusieurs éléments dont deux ont une activité satisfaisante *in vivo*. *In vivo* la résistance de *M. tuberculosis* (H37Rv) ne s'est pas manifestée durant une période de 84 jours; *in vitro* les souches résistantes présentent une virulence atténuée pour la souris.

Acknowledgment

The writers wish to acknowledge the consultations with many members of the Upjohn Research Divisions as well as the excellent technical assistance of H. Kurtz and J. Kay.

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THE EFFECT OF ISONIAZID THERAPY ON PYRIDOXINE METABOLISM IN CHILDREN[1, 2]

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(Received for publication May 5, 1956)

INTRODUCTION

Reports on the severity of the toxic manifestations observed during isoniazid therapy in man have been varied. The Committee on Therapy of the American Trudeau Society (1953) stated that "the nervous system was the most frequently involved, the commonest manifestations being twitching of the muscles, restlessness of the extremities, and hyperreflexia (1). These manifestations were not considered serious and frequently subsided even when treatment was continued. Similarly, the British Medical Research Council Report (1952) regarded isoniazid toxicity as a "very minor problem" (2). The reported side effects included increased deep reflexes, tremors of the limbs, drowsiness, twitching of the legs, difficulty of initiating micturition, increased urinary frequency, constipation, transient flushing of the face, and skin disorders.

Review of the Literature

Peripheral neuropathy seems to be the most frequently encountered side effect. Robitzek and Selikoff (3), in a group of 44 patients who received isoniazid in doses of 2 to 4 mg. per kg. of body weight and iproniazid, 2 to 10 mg. per kg., found twitching of the extremities (37 cases) and increased deep reflexes (26 cases) to be the commonest signs of toxicity. Pegum (4) observed hyperesthesia in the form of "burning feet" in a patient who received 150 mg. of isoniazid daily. Jones and Jones (5) reported the case of a patient who developed hypersensitivity of the hands and feet with considerable wasting of the palms and soles while on a dosage of 5 mg. per kg. of body weight. Lubing (6) noted pain and paresthesias in the extremities in six patients who received daily doses of 3.9 to 9.2 mg. per kg. More recently, Biehl and Vilter (7) observed peripheral neuritis in 44 per cent of their adult tuberculous patients on high doses of isoniazid (20 mg. per kg.) and in a small percentage among those on dosages as low as 3 to 5 mg. per kg. of body weight.

Serious reactions have been rare. Fetterhoff and associates (8) reported convulsions with a fatal outcome in a patient with epilepsy who received 3 mg. per kg. per day of isoniazid, and Hunter (9) reported a case of confusional psychosis with residual cerebral organic impairment in a woman who received a dose of 8 to 10 mg. per kg. of body weight.

The mechanism of isoniazid toxicity is not clear. The similarity of the chemical structure of isoniazid to that of pyridoxine suggests that it may be considered an analogue of pyridoxine. Studies have been reported, with both enzyme systems and *in vitro* bacterial

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cultures, which demonstrate the antagonism between isoniazid and pyridoxine (10–13). Lichstein (14), in further studies on the mode of competitive action of isoniazid and pyridoxine, showed that the primary site of action of isoniazid is to compete with pyridoxal phosphate for the apotryptophanase in the tryptophanase system of vitamin B_6 metabolism.

Biehl and Vilter have suggested that a pyridoxine deficiency, resulting from the antagonism between isoniazid and pyridoxine, is responsible for the neurologic manifestations observed in their series.

In the Chest Clinic of the Children's Medical Service in Bellevue Hospital, a series of tuberculous children have been treated with isoniazid. Some of these were part of a controlled study on primary pulmonary tuberculosis in which the patients to be treated were selected by random numbers. These patients received isoniazid in a dosage of approximately 5 mg. per kg. per day. In addition, a group of tuberculous children with complications, such as meningitis or miliary tuberculosis, received larger doses of isoniazid (10 to 20 mg. per kg. per day). No clinical evidence of toxicity was seen in either group.

Because of the probability that isoniazid will prevent meningitis and other serious complications of primary tuberculosis, its use routinely in this situation is advocated by many physicians. However, before embarking on a universal program of treatment of primary tuberculosis with isoniazid, it would seem wise to investigate the possible toxic reactions of isoniazid therapy in children. The present writers have been unable to find any record of such an investigation in the literature. Therefore, a study similar to that of Biehl and Vilter was planned.

The purpose of this study was to detect any clinical evidence or biochemical alteration in tuberculous children indicative of pyridoxine deficiency during isoniazid therapy. As an integral part of this investigation, other tuberculous children who were not receiving isoniazid were included to determine whether a pyridoxine deficiency state is common among tuberculous children in general.

Material

The subjects of this study are 26 tuberculous children who were admitted to the Children's Chest Service of Bellevue Hospital. There were 17 girls and 9 boys. Their ages ranged from two to thirteen years, the median age being five.

The control group was composed of 8 nontuberculous children of comparable age groups who were selected from the general pediatrics wards of Bellevue Hospital. None of them showed any manifestation of nutritional deficiency disease.

Methods

Initial examination of every patient consisted of a complete neurologic survey, hemoglobin determination, and blood cell counts.

A three-day period of study followed. An identical diet was offered for the three days of observation, and the same amount of food was consumed each day.

Tryptophane load tests were done in all the cases. L-tryptophane was given orally on the second day, the dosage being 3.0 gm. for children less than ten years old and 5.0 gm. for the others.

Serial twenty-four-hour urine specimens were collected during the three-day period, and N^1-methylnicotinamide excretion was determined, using the method of Huff and Perlzweig (15). An increase of at least 50 per cent in N^1-methylnicotinamide excretion

after tryptophane administration was considered normal, and failure of such a rise indicative of vitamin B_6 deficiency. When necessary, indwelling catheters were utilized to facilitate urine collections.

Observations

No clinical signs suggestive of pyridoxine deficiency were observed in any of the tuberculous cases in this series, irrespective of the chemotherapeutic agent received. Neurologic examinations were all within normal limits. One patient had a moderate anemia with a hemoglobin of 8.5 gm. and another failed to gain weight. As these findings had been present before the start of isoniazid therapy, it is justifiable to assume that they were unrelated to such treatment.

The responses of the 8 normal control subjects to tryptophane load tests are shown in table 1. Oral administration of L-tryptophane resulted in an increase in the urinary excretion of N^1-methylnicotinamide from a mean value of 5.28 mg. per twenty-four hours to 12.4 mg. per twenty-four hours.

The 6 tuberculous children who did not receive any isoniazid therapy and who formed a second control group showed a similar rise in N^1-methylnicotinamide after tryptophane administration (table 2). The average twenty-four-hour

TABLE 1

Urinary N^1-Methylnicotinamide Excretion in Eight Normal Children

Patient	Age	Sex	Urinary N^1-Methylnicotinamide (Mg. per 24 Hours)	
			Before L-Tryptophane	After L-Tryptophane
	years			
T.P.	9	M	8.00	16.30
L.S.	12	F	1.78	3.00
J.Z.	8	M	0.74	1.30
E.S.	9	M	6.95	14.90
E.P.	7	M	1.91	10.22
R.M.	8	M	13.70	28.50
M.V.	6	M	4.96	9.58
W.M.	6	M	4.25	15.45

TABLE 2

Urinary N^1-Methylnicotinamide Excretion in Six Untreated Tuberculous Children

Patient	Age	Sex	Urinary N^1-Methylnicotinamide (Mg. per 24 Hours)	
			Before L-Tryptophane	After L-Tryptophane
	years			
G.M.	4	M	8.45	35.70
I.M.	5	F	4.39	10.50
			3.96	15.25
E.R.	4	F	1.28	21.10
D.G.	4	M	7.18	12.80
V.R.	4	M	4.37	9.52
M.M.	10	F	3.63	26.70

urinary excretion was 4.75 mg. per twenty-four hours before the test dose and 18.8 mg. per twenty-four hours after the test dose.

The 20 tuberculous children who received isoniazid for various periods of time did not demonstrate any measurable impairment in the urinary excretion of N^1-methylnicotinamide after tryptophane load tests (table 3). The urinary excretion of this nicotinic acid derivative increased from a mean of 7.86 mg. per twenty-four hours to 20.54 mg. per twenty-four hours. These results are similar to the responses obtained in the control groups.

Of the 20 tuberculous children on isoniazid therapy, only one failed to exhibit a significant increase in the N^1-methylnicotinamide urinary excretion in response to tryptophane load tests.

The patient (*P.C.*) was a twelve-year-old adolescent girl with chronic pulmonary tuberculosis. After one month of isoniazid therapy, a load test with 5.0 gm. of tryptophane administered orally showed an initial excretion of 16.4 mg. per twenty-four hours and a

TABLE 3

URINARY N^1-METHYLNICOTINAMIDE EXCRETION IN TWENTY TUBERCULOUS CHILDREN TREATED WITH ISONIAZID

Patient	Age	Sex	Isoniazid Therapy		Urinary N^1-Methylnicotinamide (Mg. per 24 Hours)	
			Dosage	Duration	Before L-Tryptophane	After L-Tryptophane
	years		*mg/kg/day*			
A.L.	2	F	5	12 months	3.50	10.60
E.S.	5	F	5	4 months	7.70	22.00
C.F.	8	F	5	5 months	3.51	7.25
			5	6 months	16.80	35.30
L.L.	3	F	5	3 months	3.34	11.00
S.F.	3	M	5	3 months	1.62	7.40
H.R.	11	F	5	3 months	9.71	19.60
T.B.	10	F	5	1 month	4.07	8.76
F.S.	4	M	5	3½ months	3.76	10.10
H.C.	10	M	5	3 weeks	6.16	21.60
M.F.	4	M	5	27 months	4.20	26.20
R.R.	2½	M	7	6 months	3.60	7.05
C.V.	11	F	5	10 months	3.82	16.40
A.C.	13	F	5	8 months	13.60	45.00
J.W.	12	F	5	8 months	14.70	41.60
J.M.	7	M	5	2½ weeks	17.30	31.60
I.D.	12	F	5	6 days	34.60	66.70
			5	3½ months	11.80	29.00
			10	2 months	6.52	41.00
D.T.	10	F	5	6½ months	3.32	12.10
			5	7½ months	2.11	21.60
P.C.	12	F	5	1 month	16.40	14.10
			5	4 months	5.95	7.70
I.P.	6	F	7	7½ months	0.91	3.28
			7	9 months	2.65	10.30
M.C.	3½	F	20	1½ months	2.90	7.03

subsequent excretion of 14.1 mg. per twenty-four hours. The test was repeated, utilizing the same dosage after she had been receiving isoniazid for four months. Excretion increased from 5.95 mg. per twenty-four hours to 7.7 mg. per twenty-four hours, which however was not significant. A third test with a larger tryptophane dosage was contemplated but was deferred because of the patient's transfer to a sanatorium.

The meaning of the findings in this case are by no means clear, and the possibility exists that this twelve-year-old girl may have manifested a state of subclinical pyridoxine deficiency.

Discussion

Attempts to produce vitamin B_6 deficiency in adults by deprivation of pyridoxine in the diet have been unsuccessful (16). Vilter and associates (17) used the antagonist for pyridoxine, 4-desoxypyridoxine, in addition to a vitamin B-complex deficient diet in an effort to produce pyridoxine deficiency. They observed seborrheic dermatitis in twenty-nine patients, hyperpigmentation in four, glossitis in fourteen, cheilosis in nine, conjunctivitis in six, and peripheral neuritis in three. An increase in the urinary excretion of xanthurenic acid following tryptophane loading and a restoration to normal values following pyridoxine administration were observed in all of the patients who developed these lesions.

In children, pyridoxine deficiency is not manifested by peripheral nerve involvement or by skin changes. The occurrence of convulsions, hypochromic anemia, and weight loss were noted by Snyderman and co-workers (18) in two infants placed on pyridoxine-deficient diets. Coursin (19) and Hunt and associates (20) reported recurring convulsive seizures relieved by the administration of pyridoxine in an infant. A difference in response to pyridoxine deficiency between the two age groups seems plausible. However, it is questionable whether the use of the antagonist, desoxypyridoxine, to induce the deficiency in adults produces a true picture of pyridoxine deficiency.

The tryptophane load test was utilized as a measure of vitamin B_6 deficiency. The conversion of tryptophane to nicotinic acid has been studied in many animal species (21–24), as well as in neurospora (25), and all evidence points to its dependence on kynureninase, a vitamin B_6 containing enzyme. A deficiency of vitamin B_6 results in failure to convert kynurenine. This can be detected chemically in two ways: by the accumulation of metabolic products from steps proximal to the block and resulting lateral reactions (notably by the accumulation of xanthurenic acid); and by the failure of metabolic products to appear from steps beyond the block (N^1-methylnicotinamide and its pyridone derivative are those usually tested for). The significance of both types of reaction is the same; in both instances kynureninase is the limiting factor. The writers have preferred using the latter as a means of detecting vitamin B_6 deficiency.

On the basis of the present results, it appears that, contrary to experimental findings in adults, children on isoniazid therapy on dosages of 5 to 20 mg. per kg. per day, and for as long a period as twenty-seven months, do not develop pyridoxine deficiency. However, because of the small number of patients in the series who received large doses of isoniazid, a further study on high dosage seems indicated.

Summary

The incidence of pyridoxine deficiency was investigated in a group of 26 tuberculous children. Of these, 20 received isoniazid therapy for various periods. Clinical signs of deficiency, such as convulsions, anemia, or weight loss, were not observed.

The ability to convert tryptophane to N^1-methylnicotinamide was used as a biochemical test for pyridoxine deficiency. Except in one case, all patients showed an increase in urinary N^1-methylnicotinamide excretion following tryptophane loading, thus confirming the absence of pyridoxine deficiency.

In contrast to the situation in adults, isoniazid in the dosage used did not induce a pyridoxine deficiency in children. It appears, therefore, that prophylactic administration of pyridoxine during isoniazid therapy, as advised for adults, is unnecessary in children.

Sumario

El Efecto de la Isoniacidoterapia sobre el Metabolismo de la Piridoxina en los Niños

En un grupo de 26 niños tuberculosos se investigó la incidencia de la deficiencia de piridoxina. De ellos, 20 recibieron isoniacidoterapia durante períodos variables, sin que se observaran signos clínicos de deficiencia, tales como convulsiones, anemia o pérdida de peso.

Como ensayo bioquímico para la deficiencia de piridoxina se utilizó la capacidad para convertir el triptófano en N^1-metilnicotinamida. Aparte de un caso, todos los niños mostraron aumento de la excreción urinaria de N^1-metilnicotinamida después de cargar con triptófano, confirmando así la falta de deficiencia de piridoxina.

En contraposición a su efecto en los adultos, la isoniacida no provoca deficiencia de piridoxina en los niños. Parece, pues, que la administración profiláctica de piridoxina durante la isoniacidoterapia, según se recomienda para los adultos, es innecesaria en los niños.

Resume

L'effet de l'isoniazidothérapie sur le métabolisme de la pyridoxine chez l'enfant

La fréquence de la carence en pyridoxine fut recherchée dans un groupe de 26 enfants tuberculeux. Parmi ceux-ci, 20 furent soumis à l'isoniazidothérapie pendant des périodes variées. Les signes cliniques de déficit, tels que les convulsions, l'anémie ou l'amaigrissement ne furent pas observés.

L'aptitude à convertir le tryptophane en N^1-méthylnicotinamide a été utilisée comme test biochimique de la carence en pyridoxine. A l'exception d'un cas, tous présentaient une augmentation de l'élimination urinaire de N^1-méthylnicotinamide après l'ingestion d'un excédent de tryptophane, ce qui confirmait l'absence d'une carence en pyridoxine.

En contraste avec son action chez l'adulte, l'isoniazide n'entraîne pas la carence en pyridoxine chez l'enfant. En conséquence, l'administration préventive de pyridoxine au cours de l'isoniazidothérapie, préconisée chez l'adulte, n'est pas nécessaire chez l'enfant.

Acknowledgment

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STUDY OF TUBERCULIN PROTEIN AND POLYSACCHARIDE ANTIGENS BY GEL-DIFFUSION TECHNIQUE[1, 2]

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INTRODUCTION

Several proteins and at least two polysaccharides with characteristic chemical and electrochemical properties have been repeatedly isolated from unheated culture filtrates of tubercle bacilli or from live bacillary bodies by Seibert and co-workers (1–3).

All of these fractions were antigens (4). Some were antigenic, since they were able to cause the production of antibodies when injected into rabbits, as well as to give precipitin and complement-fixation reactions with antisera. Others, especially the low molecular weight polysaccharide, could merely react with antisera and would, therefore, be classed as haptenes.

While the purest proteins so far isolated appeared as single components in electrophoresis (2), nevertheless the curves were not always strictly homogeneous and, moreover, cross precipitin reactions between the antigens have been found. It was desirable, therefore, to study the reaction of these antigens by the method of diffusion in gels, since by this highly sensitive method one would be able to detect the number of antigens present in each fraction. Such studies were made, and the results obtained are reported in this paper.

MATERIALS AND METHODS

Antigens: Protein fractions representative of the different types of tuberculin proteins, designated as *A*, *B*, and *C*, and whose properties and methods of preparation were given earlier (1, 2), were used as antigens. The samples tested were as follows: *A* (107 II), *B* (109 II), and *C* (108 I). A protein isolated (3) directly from live tubercle bacilli (109 BId) by extraction with solid urea and another protein, PPD-S, isolated from a heated culture filtrate were also used. The latter was the product adopted as the International Standard for tuberculins (5).

The polysaccharide preparation used was known as *Type I*, a small molecular product isolated from the filtrate of a half-saturated ammonium sulfate precipitate of a heated culture filtrate (6). It had 0.27 per cent nitrogen.

All protein antigens were diluted with buffered saline to final concentrations of 3.0 and 0.5 mg. per ml., and sufficient merthiolate was added to give a final concentration of 0.01 per cent or 1:10,000 merthiolate. In the case of the polysaccharide a much lower concentration (0.062 and 0.0075 mg. per ml.) was found to be optimal.

All of these products had been found to serve as good antigens in the precipitin reaction (4). Moreover, all were able to elicit antibodies when injected into the rabbit, with the

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TABLE 1

Number and Position of Precipitation Bands in Agar Layer

Mm. from Serum Meniscus	III6 = 51						III6 = 52						AD5 = 33						AD5 = 34					
	Polysaccharide	A	B	C	Bacillary	PPD-S	Polysaccharide	A	B	C	Bacillary	PPD-S	Polysaccharide	A	B	C	Bacillary	PPD-S	Polysaccharide	A	B	C	Bacillary	PPD-S
10			Tr*																					
9																								Tr
8					Tr												+							
7					Tr						++													+
6				+s		++			Tr			++				+		+					+	
5			+	+s			++	+	+						++	+				++	++	++s		Tr
4				+s				++s	+s	++	Tr			+s		+								
3			+	+						+		+		+s						Tr	+	+		
2	++	++	+			++	+++		+						+			+	+++					
1								++	+				++		Tr	++				+		++		
0	+	++	+	++	+s	+s	+		+	+	+	+		++	++s		+	+	+	+	++		+	+
	No. 17						No. 43						No. 46						No. 84					
10																								
9																								
8			++													+	++							
7					+	++			Tr		+s							++						
6				++		++																		
5		++	+					Tr	+s	++s	Tr							+++						
4	+++			++s				+						+++		+								
3		+	++					Tr		+			+++++		+++++	+								
2									Tr					+				++						
1	++	+	++	++s			+	++	+	++	Tr	+				+	+	+						
0					+	+							+++	++	+++s					Tr	Tr			

* Tr = trace; s = sharp

exception of the polysaccharide. This was effective merely as a haptene. Three of the preparations, *C*, PPD-S, and bacillary protein, were also able to serve as antigens in the complement-fixation test (4).

Sera: Sera were taken from the blood of rabbits previously sensitized by repeated injections with BCG or with the different fractions just described, and were used undiluted. More detailed treatment of the rabbits can be found in a previous paper (4).

Agar: The agar was prepared as suggested by Harris (7) by immersing commercially available agar (Difco®) in several volumes of cold distilled water, stirring, and centrifuging in a refrigerated centrifuge. This process was repeated several times. Instead of lyophilizing, the sedimented agar was mixed with 0.85 per cent sodium chloride to make approximately a 0.6 per cent solution. It was stirred in a boiling water bath and autoclaved at 15 pounds of pressure for fifteen minutes. While the solution was still hot, sufficient merthiolate was added to give a final concentration of 1:10,000. After mixing well, moisture determinations were made on the mixture and also on 0.85 per cent sodium chloride solution so that the actual concentration of the agar in the solution could be calculated, and from this a 0.3 per cent solution was made for use in the experiments.

Method

The method of double diffusion described by Oakley and Fulthorpe (8) and modified by Harris and associates (9) was used. Tubes 10 cm. in length were lined with the 0.3 per cent agar solution described above and then the antibody-containing serum was put into them to a 2 cm. mark from the bottom. The agar solution, at 48°C., was carefully layered over the serum, avoiding bubbles, to a 3 cm. mark, and when this had solidified, the antigen was layered on top of the column to a 5 cm. mark. The tubes were then sealed with parafilm and placed in a glass jar in a closed cabinet at constant temperature (25°C.). The maximal number of bands appeared at three to five days when record was made of the density as pluses, of the sharpness (noted with an *s*), and of the distance in centimeters from the serum-agar interface.

Results

In the case of most of the fractions studied many bands appeared, indicating the presence of more than one antigen. The data are assembled in table 1. For each antigen, the intensity of the bands of precipitation are recorded as pluses and their positions in the agar are noted. When the bands were sharp, an *s* was added.

The maximal number of bands is recorded, regardless of the dilution of antigen used (3.0 or 0.5 mg. per ml. for the proteins, or 0.062 or 0.0075 mg. per ml. for polysaccharide).

All the rabbits mentioned in table 1, except *Nos. 48* and *84*, had been injected repeatedly with live BCG organisms; *No. 48* had received dead BCG bacilli, and *No. 84* was a normal rabbit. Rabbit *No. 43* had shown very little antibodies to the polysaccharide by the usual precipitin technique (4).

Several bands appeared in the case of most of the protein antigens, except the bacillary protein, in which only two distinct bands were usually found. It is also evident that the bands which appeared high in the agar layer or near the antigen-agar interface (at 7 to 9 mm.) were to be found only in the tubes containing *B*, *C*, PPD-S, or bacillary protein antigens. These four fractions also had in common the fact that they contained a protein component with a high electrophoretic mobility. Moreover, all of them, except *B* protein, were excellent complement-fixing antigens (4).

TABLE 2

EFFECT OF DILUTION OF POLYSACCHARIDE ON ZONES OF PRECIPITATION

Mm. from Serum Meniscus	Dilution of Polysaccharide (mg. per ml.)											
	3.0	1.0	0.5	0.25	0.125	0.062	0.031	0.015	0.0075	0.00375	0.00187	0.00093
10												
9												
8												Tr*
7												
6											+	
5										+++		
4									++++			
3								+++				
2						++++	+++++					
1					++++				++	++	+	+
0	+	+	++	+++			+	++				
−1						+						
−2					++							
−3				+								
−4			Tr									
−5	Tr											
−6		+										
Ppt.†		Ppt.	Ppt.	Ppt.	Ppt.							

* Tr = trace

† Ppt = precipitate

The *A* protein fraction in all cases showed several bands, which occupied intermediate positions in the agar.

The positions of the protein bands may have significance for their specificity, since the concentration of the proteins was the same in all cases (3.0 and 0.5 mg. per ml.). At higher dilutions some of the bands, especially the higher ones, seemed to disappear.

Polysaccharide gave a band which always occurred at or close to the interface between serum and agar. A second definite band also appeared as the polysaccharide antigen was diluted, and this band occurred progressively higher in the column with further dilution. (The bands with antiserum of rabbit *No. 17* are shown in table 2.) At the higher concentrations of polysaccharide this band was visible below the interface between agar and serum and may even have formed a part of the precipitate which often appeared. In great antigen excess (3.0 mg. per ml.) the antigen-antibody complexes were obviously in solution. It is noteworthy, therefore, that a much higher dilution of polysaccharide antigen was optimal for precipitation of both of these bands than with any of the protein antigens, and this was consistent with results obtained previously by the usual precipitin technique (4).

When normal serum (*No. 84*) was studied with the same antigens no significant bands occurred with any of them.

Discussion

The method of double diffusion into a gel, which was used in this study, appears to be very sensitive for determining the purity of antigens. The fact that most of the tuberculin antigens studied showed two, three, or more bands with BCG antisera indicates that they consisted of mixtures of different antigens.

The band or bands for tuberculopolysaccharide were readily distinguishable as the ones at or close to the interface between serum and agar at the concentrations used in these experiments. Their distinctive feature was that much higher dilution of the antigen was required for optimal precipitation than for the protein antigens. This paralleled the experience with this antigen in the usual precipitin technique, as well as in the complement-fixation test (4). It is accordingly easy to understand why a band appeared in this position with every antigen studied, since a dilution of the protein antigens, even to a concentration of 0.5 mg. per ml., could carry with it sufficient polysaccharide to give a band, even if it had been an impurity to the extent of as little as 1.5 per cent. For the same reason it would be possible to detect a minute amount of antibody to polysaccharide in any serum, as was the case with rabbit *No. 43*.

It is also possible that some of the bands in protein antigen-containing tubes which were slightly higher than the one at the interface may be due to the second component found in the purified polysaccharide. Whether the two bands observed represent different polysaccharides, or the second one some other impurity, remains for further study.

Other bands for protein appeared higher up in the column and further purification of the antigens is indicated. It was evident that the *C*, bacillary protein, and PPD-S antigens contained a component which may not be present in the *A* protein. This component apparently gave rise to a band close to the antigen-agar interface (at 7 to 9 mm.).

The multiplicity of antigens here demonstrated to be present in the tuberculin made from a single strain of tubercle bacillus indicates the tremendous task involved in a study of the relationships of different strains, such as that reported by Parlett and Youmans (10). Their ability to group the acid-fast organisms indicates that isolation of group-specific antigens should be possible.

Summary

The method of double diffusion into gels in one dimension has proved very helpful in the study of tuberculin protein and polysaccharide antigens.

The polysaccharide gave two distinct bands with strong BCG antiserum and there was always a band at or close to the interface between serum and agar. Both bands were distinguishable by the fact that their optimal precipitation occurred at a much higher dilution of the polysaccharide (0.062 to 0.0075 γ per ml.) than was true with the protein antigens. Therefore, very minute amounts of polysaccharide antigens as well as antibodies to polysaccharide can be detected by this sensitive method.

The optimal precipitation with the tuberculin protein antigens occurred at a concentration of 3.0 or 0.5 mg. per ml. *Type A* protein showed several bands

which appeared no higher than the middle of the agar layer. *Types B* and *C*, bacillary protein, and PPD-S also showed several bands, but with these antigens bands were also frequently found close to the interface between agar and antigen.

Sumario

Estudio de los Antígenos de la Proteína y los Polisacáridos de la Tuberculina con la Técnica de la Difusión en Geles

El método de la doble difusión en geles en una dimensión ha resultado muy útil en el estudio de los antígenos de proteína y de polisacáridos de la tuberculina.

El polisacárido produjo dos bandas bien definidas con antisuero BCG potente, habiendo siempre una en la intercara entre el suero y el agar o cerca de ella. Ambas fajas eran distinguibles por tener lugar su preciptación óptima a una dilución mucho más alta (0.062 a 0.0075 mg. por ml.) del polisacárido que la observada con los antígenos de proteína. Por lo tanto, pueden distinguirse con este método delicado cantidades muy pequeñas de antígenos de polisacáridos así como anticuerpos de éstos.

La precipitación óptima con los antígenos de proteína tuberculínica ocurrió a una concentración de 3 ó 0.5 mg. por ml. La proteína de *tipo A* mostró varias bandas que no subieron más allá del medio de la capa de agar. Los *tipos B* y *C*, proteína bacilar y el PPD-S también mostraron varias bandas, pero con estos antígenos las bandas también quedaban frecuentemente próximas a la intercara entre el agar y el antígeno.

Resume

Etude des antigènes de la protéine et du polysaccharide de la tuberculine par la technique de diffusion dans le gel

La méthode de la double diffusion dans les gels en une dimension s'est montrée très utile dans l'étude des antigènes de la protéine et du polysaccharide de la tuberculine.

Le polysaccharide a donné deux bandes distinctes avec l'antisérum BCG fort et on observait toujours une bande située au niveau ou à proximité de la couche interface. Ces deux bandes se distinguaient par le fait que leur précipitation optima se produisait à une dilution plus élevée lorsqu'il s'agissait du polysaccharide (0,062 à 0,0075 mg/ml) que pour les antigènes de la protéine. En conséquence, cette méthode sensible permet de déceler des quantités minimes d'antigène du polysaccharide ainsi que les anticorps du polysaccharide.

La précipitation optima des antigènes de la protéine de la tuberculine se produisait à la concentration de 3 ou de 0,5 mg/ml. La protéine du *type A* présentait plusieurs bandes ne dépassant pas le milieu de la couche agar. Les *types B* et *C*, la protéine bacillaire et PPD-S présentaient également plusieurs bandes, mais avec ces antigènes les bandes étaient aussi observées au voisinage de la couche interface entre l'agar et l'antigène.

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THE PROPERTIES OF PARA-AMINOSALICYLIC ACID-RESISTANT MYCOBACTERIUM TUBERCULOSIS VAR. HOMINIS[1,2]

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Introduction

The antituberculous activity of para-aminosalicylic acid (PAS) was first described by Lehmann (1). Strains of *M. tuberculosis* var. *hominis* which are resistant to isoniazid or streptomycin have been found susceptible to PAS (2, 3). Furthermore, the effect of PAS in delaying the emergence of resistance to other antituberculous agents has been demonstrated both *in vivo* and *in vitro* (4, 5). Thus PAS has maintained a role as one of the most important antituberculous agents.

The occurrence of PAS-resistant strains of *M. tuberculosis* var. *hominis* is now well recognized. These resistant strains develop during administration of PAS alone or in combination with isoniazid or streptomycin. Relatively little information regarding the characteristics of these PAS-resistant mutants and of the effects of PAS upon these mutants is available. It is the purpose of this paper to describe certain *in vitro* observations on the properties of PAS-resistant mutants of *M. tuberculosis* var. *hominis*.

Materials and Methods

M. tuberculosis var. *hominis*, strain Aoyama *B*, was used as the test organism throughout this study. Ogawa's medium had been used to maintain this strain with monthly passage and was also the medium used for all tests in this study (6).

Composition of Ogawa's medium:

KH_2PO_4	1.0 gm.
Sodium glutamate	1.0 gm.
Distilled H_2O	100.0 ml.
Eggs (whole)	200.0 ml.
Glycerol	6.0 ml.
2 per cent malachite green	6.0 ml.

Preparation of the medium: Ten milliliters of medium were poured into each tube and the medium was heated to 85° to 87°C. for forty minutes for sterilization. The antituberculous agents were dissolved in distilled water and the appropriate dilutions of PAS[3] and combinations of PAS with dihydrostreptomycin[4] and isoniazid[5] were made in the medium

[1] From the Obuso National Sanatorium, Obu, Chita, Aichi, Japan.

[2] The original manuscript, submitted to the Review by Dr. Tsukamura, was a detailed presentation of the observations described in the present report. Because of space limitation, it was not possible to publish the manuscript as originally submitted. Accordingly, with Dr. Tsukamura's permission, the original manuscript was turned over to Dr. Thomas F. Sellers, Jr., of the School of Medicine, Emory University, Atlanta, Georgia, for reorganization and condensation. Both the author of the manuscript and the Editor of the Review wish to express their appreciation to Dr. Sellers for his very considerable contribution to the publication of this report.

[3] The sodium PAS used in this study was manufactured by Tanabe.

[4] The dihydrostreptomycin sulfate used in this study was manufactured by Meiji.

[5] The isoniazid used in this study was manufactured by Takeda.

TABLE 1

SUSCEPTIBILITY TO PAS OF THE PARENT DRUG-SUSCEPTIBLE STRAIN AND THE PAS-RESISTANT STRAIN

γ of PAS per Ml. of Medium	Susceptible Parent Strain*	γ of PAS per Ml. of Medium	PAS-Resistant Strain†
20.00	—	800.00	—
10.00	—	400.00	3+
5.00	—	200.00	3+
2.50	—	100.00	4+
1.25	2+	50.00	4+
0.63	3+	25.00	4+
0.32	4+	12.50	4+
0.16	4+	6.25	4+
0.08	4+	3.13	4+
0.04	4+	1.57	4+
0 (control)	4+	0 (control)	4+

* Each inoculum per tube contained 1.6×10^6 viable cells.
† Each inoculum per tube contained 1.3×10^6 viable cells.
Growth was observed after six weeks incubation:
— No growth.
1+ 1–10 colonies.
2+ 11–100 colonies.
3+ More than 100 colonies but not confluent.
4+ Growth confluent.

before sterilization. The activities of PAS and isoniazid were not regarded as altered by sterilization. Because of the decreased activity of dihydrostreptomycin resulting from adsorption of the drug on coagulated egg protein (6), the activity of this agent was considered to be one-half of the original concentration.

PAS-resistant strains were isolated after multiple transfers of the drug-susceptible parent strain in the presence of PAS. Growth of the parent strain was partly inhibited by 0.64 γ of PAS per ml. but not by 0.32 γ per ml. The resistant strain used in this study was obtained from medium which contained 100 γ of PAS per ml. and was maintained by successive transfers on similar drug medium three times during every six-week period of incubation.

RESULTS

Composition of the PAS-resistant population: The results of *in vitro* tests of PAS susceptibility of the parent-susceptible strain and of the PAS-resistant population may be seen in table 1. From these data it appears that the level of total resistance to the effects of PAS is 0.32 γ of drug per ml. for the parent strain and 100.0 γ per ml. for the resistant population. If the composition of these populations were homogeneous, such levels obtained with the use of the large inocula of viable cells would undoubtedly indicate true resistance. A heterogeneous population, however, would not allow an accurate determination of total resistance on a single examination. Since each control slant will support approximately one thousand colonies, such luxuriant growth would be observed on medium containing a given amount of PAS if one thousand resistant cells contained in the much larger total inoculum were evenly distributed over the medium.

TABLE 2

COMPOSITION OF THE PAS-RESISTANT STRAIN

γ of PAS per Ml. of Medium	Average Number of Colonies	
		per cent
0 (control)	216.0 ± 70.9	100
1	85.0 ± 12.5	39
10	56.5 ± 15.8	26
100	58.1 ± 14.2	27

Growth was observed after six weeks' incubation.

Inoculum for each tube = 0.03 ml. of a 10^{-4} cell suspension (0.0001 mg. cells per ml. wet weight).

TABLE 3

PAS-RESISTANT STRAIN: PERCENTAGE OF MUTANTS RESISTANT TO 100 γ OF PAS PER ML.

Experiment Number	Number of Tubes Used	Average Number of Viable Cells in Medium Without PAS	Average Number of Drug-Resistant Mutants	Percentage of Mutants
A	10	170.7 ± 24.7	93.5 ± 16.4	54.9
B	10	134.1 ± 27.0	63.8 ± 13.6	47.5
C	10	119.0 ± 31.2	16.0 ± 5.3	20.4
D	10	282.1 ± 51.2	37.8 ± 10.8	13.4
E	10	20.0	2.2	11.0

Growth was observed after six weeks' incubation.

With respect to this consideration, the composition of the PAS-resistant population was subjected to analysis, and a relatively heterogeneous population was demonstrated. As may be seen in table 2, a considerable number of individual cells susceptible even to 1 γ of PAS occurred among the PAS-resistant population. It should be recalled that the PAS-resistant inocula had been grown for eighteen weeks on medium which contained 100 γ of PAS per ml., with three successive transfers on similar fresh medium in each six-week period of incubation. Therefore, heterogeniety would appear to be one characteristic of a PAS-resistant strain derived in this manner.

The heterogeneous composition of a PAS-resistant strain was further suggested by the following experiment. The above-tested resistant strain was inoculated in five separate experiments on medium which contained 100 γ of PAS per ml. and was incubated for six weeks at 37°C. The growth was harvested and the composition of the populations was assayed on control medium and medium which contained 100 γ of PAS per ml. The percentage of the average number of drug-resistant mutants to the total number of viable cells was thereby determined. It may be seen in table 3 that the number of mutants resistant to 100 γ of PAS per ml. was relatively unstable and varied from 11 to 55 per cent of the total population.

Effect of PAS upon rate of growth of PAS-resistant mutants: Once again the PAS-resistant strain was inoculated into non-drug medium and incubated at

TABLE 4

RELATIONSHIP BETWEEN NUMBER OF VISIBLE COLONIES AND INCUBATION PERIOD

PAS Concentration	Inoculum Size*	Average Number of Colonies		
		Weeks of Incubation		
		4	5	6
γ per ml.				
0	10^{-4}	21.2	21.6	
0.5	10^{-4}	0.2	8.2	14.0
1.0	10^{-4}	0	17.0	16.8
10.0	10^{-2}	108.6	226.0	277.0
100.0	10^{-2}	90.6	150.6	Ca. 300
500.0	10^{-2}	32.8	59.0	79.6

* Dilution of original cell suspension

37°C. for four weeks. Appropriate dilutions of the cell suspensions harvested therefrom were inoculated into six different concentrations of PAS with ten slants in each series. Growth at 37°C. was observed and recorded after four, five, and six weeks of incubation. Results are shown in table 4. After four weeks of incubation, numerous well-developed colonies were observed on the control slant, and thereafter the number of colonies did not change. However, during the same four-week period, virtually no colonies developed on medium containing PAS which had been inoculated with a 10^{-4} dilution of the cell suspension. Visible small colonies were observed only after five weeks and well-developed colonies only after six weeks of incubation. Furthermore, the diameter that the colonies attained was progressively smaller with each higher concentration of PAS in the medium. The diameter was greatest on control medium and smallest on medium containing 1 γ of PAS.[6]

However, when a much larger inoculum was used, as in the series inoculated with a 10^{-2} dilution shown in table 4, moderately well-developed colonies were observed after four weeks even in the presence of much greater PAS concentrations. Here again, the number of visible colonies increased with the period of incubation at each PAS concentration. The diameter of the colonies was greatest on medium containing 10 γ of PAS and smallest on medium containing 500 γ of PAS.

The appearance of colonies in medium containing the higher concentration of PAS was much earlier than in medium of low PAS concentration. This paradox is explained by the fact that inocula of different sizes were used. It is thought that agglutination of cells occurred in the 10^{-2} dilution, and clumps of these cells were

[6] It seems unlikely that the increase in the number of colonies in the later stages of incubation was derived through progressive decrease of PAS in the medium. Duplicate series of PAS medium at the various concentrations were prepared and one set was incubated; the other was refrigerated for the above periods. Thereafter, all series were inoculated with inoculum of the same size and were incubated for four, five, and six weeks at 37°C. This biologic assay indicated that at least one-fourth of the original PAS activity remained at the end of the six-week period.

TABLE 5

HEREDITARY TRANSMISSION OF PAS RESISTANCE

Source of Inoculum	The Number of Tubes Showing Growth on Medium Containing 100 γ of PAS per Ml.					
Single colony derived from single cell of the PAS-resistant strain*	No growth	1+	2+	3+	4+	total
	0†	1	21	37	71	130
	130	130	130	130	130	130
Single colony derived from single cell of the parent susceptible strain	No growth	1+	2+	3+	4+	total
	100	0	0	0	0	100
	100	100	100	100	100	100

* Colonies used in the experiment were those grown on controls in the experiment shown in table 4.

† Numerator = number of tubes with growth.

inoculated; colonies produced from these clumps of agglutinated cells appeared earlier than colonies derived from single cells of the 10^{-4} dilution.

Hereditary transmission of PAS resistance: As previously shown, the population of the PAS-resistant strain was very heterogeneous. A number of the cells in the population behaved as if they were susceptible to PAS. The question arose whether these represented reverse mutation of PAS-resistant cells. If a number of truly susceptible cells, not only phenotypically but also genotypically, could be developed by reverse mutation, colonies formed by these cells should be susceptible to PAS.

In view of these considerations, 130 colonies developed from single cells of the PAS-resistant strain which had grown on medium without PAS and 100 colonies developed from single cells of the susceptible parent strain were selected at random. One loopful of each colony was inoculated onto medium which contained 100 γ of PAS per ml. and incubated for eight weeks at 37°C. As may be seen in table 5, all tubes which had been inoculated with a single colony of the PAS-resistant strain showed growth, varying from a few colonies per tube to abundant pellicle formation. In contrast, no visible growth was obtained even after eight weeks' incubation in the tubes inoculated with the susceptible parent strain.

These results indicate that almost all individual cells of the PAS-resistant population had the property of hereditary PAS resistance. Therefore, even if a number of individual cells of the PAS-resistant strain appeared phenotypically susceptible, it must be considered that they contained the latent hereditable property of PAS resistance, i.e., the hereditable property which produces increased rate of mutation to higher levels of PAS resistance.

In view of the results shown in tables 2, 3, 4, and 5, the rate of appearance of mutants resistant to higher levels of PAS concentration seems to be decreased by the transfer of the resistant strain in the absence of PAS. The strain used in the

experiment shown in table 4 had grown on non-drug medium for six weeks, whereas the strain used in table 5 had grown on non-drug medium for twelve weeks with two transfers. Subsequently a third transfer to another series of non-drug medium was made. Ten colonies were taken at random and individual cell suspensions were prepared from each colony. Approximately the same amount of each suspension (0.03 ml. of 0.1 mg. wet weight of cells per ml. of saline) was inoculated on control medium and medium which contained 100 γ of PAS per ml. Although the controls showed confluent growth after four weeks of incubation, the number of colonies grown on the drug medium varied from several to 100. Therefore, the strain used in this experiment appeared to contain a much smaller number of highly resistant cells. This raises the possibility that the proportion of PAS-resistant cells in a given population is decreased by repeated transfer of a resistant strain on non-drug medium. Further experiments to substantiate this possibility are planned.

In vitro effect of PAS combination with dihydrostreptomycin or isoniazid on the PAS-resistant strain: As may be seen in tables 6 and 7, the inhibition of growth of the PAS-resistant strain by dihydrostreptomycin or isoniazid was increased significantly by the addition of PAS. This effect appeared to be more striking with dihydrostreptomycin than with isoniazid.

Two possibilities are considered in the mechanism of the PAS effect in these combinations. One is that the addition of PAS causes a relative decrease in viable cells. This actually appeared to occur since a decrease was noted in the number of cells growing out from the heterogeneous population as well as a delay in growth of the population as a whole. The other possibility is a decreased mutation to streptomycin resistance of the PAS-resistant strain in the presence of PAS. No further study of this possibility was made.

Discussion

The results described in the present paper indicate that heterogeneity is a characteristic of a PAS-resistant strain. The heterogeneous character is not attributable to incomplete selection since the heterogeneity was observed even in the strain repeatedly selected by higher concentrations of PAS. Moreover, all single colonies grown on non-drug medium developed a number of mutants highly resistant to PAS. Even when a number of individual cells appeared to have decreased resistance to PAS, highly resistant cells were found in their descendants. These findings suggest that the genotype of PAS resistance is maintained in cells grown on non-drug medium although the phenotype of PAS resistance in the cells may appear to have been diminished. In view of these results, especially the heterogeneity of the PAS-resistant strain, the possibility is suggested that multiple genes are related to PAS resistance.

It would appear that the proportion of mutants highly resistant to PAS in the population is decreased by repeated transfer of the PAS-resistant strain on non-drug medium and that the tendency of reverse mutation towards drug susceptibility is relatively significant. If this be true, decrease in PAS resistance would follow interruption of clinical administration of PAS. Nevertheless, adminis-

tration of PAS again would probably produce a reappearance of resistant cells in a relatively short period since the property of increased mutation to resistance would be maintained even during the interruption of therapy.

The *in vitro* effect of PAS on the enhancement of the antituberculous action of dihydrostreptomycin or isoniazid seemed to be derived from the PAS effect in delaying growth of the PAS-resistant strain as well as in reducing the number of individual cells of the heterogeneous population. That these results are characteristic of the PAS-resistant strain is further substantiated by the experience with the relatively homogeneous, dihydrostreptomycin-resistant and isoniazid-resistant bacterial populations. In each instance the tuberculostatic action of either agent has not been enhanced by addition of the drug to which the population was resistant. Therefore, the effect of PAS in such combinations appears to be a characteristic of the PAS-resistant strain and one of considerable clinical significance.

Although the delay in growth of the PAS-resistant strain and the inhibition of a number of individual cells may also occur *in vivo* when PAS is administered alone, the effect would not be expected to be great since the *in vivo* tuberculostatic effect of PAS is weak and also because these effects are significantly decreased in the presence of a large number of PAS-resistant cells. Nevertheless, the combined effect of PAS and dihydrostreptomycin or isoniazid would be more striking than the effect of PAS alone. As may be seen in tables 6 and 7, combination with these

TABLE 6

EFFECT OF PAS ON THE TUBERCULOSTATIC ACTION OF DIHYDROSTREPTOMYCIN AGAINST THE PAS-RESISTANT STRAIN WHEN USED IN COMBINATION

Dihydrostreptomycin	PAS 0 (Control)	PAS	PAS
γ per ml.		*1 γ per ml.*	*10 γ per ml.*
20.00	—	—	—
10.00	—	—	—
5.00	—	—	—
2.50	2+	—	—
1.25	3+	1+	—
0.63	4+	2+	—
0.32	4+	3+	1+
0.16	4+	4+	2+
0.08	4+	4+	2+
0.04	4+	4+	2+
0.02	4+	4+	3+
0	4+	4+	4+

Growth was observed after six weeks' incubation. The inoculum contained 1.34×10^6 viable cells per tube of the PAS-resistant strain.

The composition of population was as follows:

The average number of colonies grown on medium containing no drug was 134.1 ± 27.0.

The average number of colonies grown on medium containing 10 γ of PAS per ml. of medium was 81.8 ± 10.3.

The average number of colonies grown on medium containing 100 γ of PAS per ml. of medium was 63.8 ± 13.6.

TABLE 7

EFFECT OF PAS ON THE TUBERCULOSTATIC ACTION OF ISONIAZID AGAINST THE PAS-RESISTANT STRAIN WHEN USED IN COMBINATION

Isoniazid	PAS 0 (Control)	PAS	PAS
γ per ml.		*1 γ per ml.*	*10 γ per ml.*
1.000	—	—	—
0.500	—	—	—
0.250	—	—	—
0.125	1+	—	—
0.063	1+	1+	—
0.032	4+	1+	—
0.016	4+	3+	1+
0.008	4+	3+	1+
0.004	4+	4+	3+
0.002	4+	4+	4+
0	4+	4+	4+

Growth was observed after six weeks' incubation. The inoculum used was the same as that used in the experiment shown in table 6.

drugs produced an enhanced effect, although the effect of PAS alone was very slight. It seems logical that these results should also apply *in vivo* to PAS-resistant strains. Therefore, it is considered that, even in the presence of PAS resistance, clinical administration of PAS when in combination with dihydrostreptomycin or isoniazid should not be interrupted unless evidence of resistance to both agents is present.

SUMMARY

The properties of para-aminosalicylic acid (PAS)-resistant *Mycobacterium tuberculosis* var. *hominis* (strain Aoyama *B*) have been studied.

The population of a PAS-resistant strain was significantly heterogeneous and a decrease in the degree of PAS resistance in a number of individual cells of the population was observed. Even when PAS resistance of individual cells was phenotypically decreased, the genotype of PAS resistance, i.e., the hereditable property producing increased rate of mutation to higher levels of PAS resistance, was maintained.

An *in vitro* effect of PAS in delaying the growth of all individual cells of the PAS-resistant strain was present, and this effect was demonstrable only when the number of viable cells inoculated was relatively small.

It is suggested that the proportion of mutants highly-resistant to PAS in the population is decreased by repeated transfer of the PAS-resistant strain to PAS-free medium and that the tendency to reverse mutation toward susceptibility is relatively significant. It is also suggested that multiple alleles are related to PAS resistance.

The *in-vitro* combined effect of PAS in enhancing the tuberculostatic action of dihydrostreptomycin or isoniazid against the PAS-resistant strain has been observed and the mechanism of the effect has been discussed. In view of these re-

sults it is suggested that, even in the presence of PAS resistance, clinical administration of PAS in combination with dihydrostreptomycin or isoniazid is more effective than administration of dihydrostreptomycin or isoniazid alone. Therefore, it would seem that clinical administration of PAS should not be interrupted because of the emergence of PAS resistance, but administration of PAS in combination with streptomycin or isoniazid, especially with streptomycin, should be continued unless double resistance occurs.

Summario

Las Propiedades del Mycobacterium Tuberculosis var. Hominis PAS-Resistente

Estúdianse las propiedades del *Mycobacterium tuberculosis* var. *hominis* (cepa Aoyama B) resistente al ácido para-aminosalicílico (PAS).

La población de la cepa PAS-resistente es significativamente heterogénea, observándose disminución de la intensidad de la PAS-resistencia en varias distintas células de la población. Aun cuando disminuye fenotípicamente la PAS-resistencia de distintas células, se mantiene el genotipo de dicha resistencia, es decir, el mayor índice heredable y productor de propiedades de mutación a niveles más altos de PAS-resistencia.

Hállase presente el efecto *in vitro* del PAS de demorar la proliferación de todas las distintas células de la cepa PAS-resistente, considerándose este efecto importante únicamente cuando era relativamente pequeño el número de células viables inoculadas.

Se ha sugerido que la proporción de mutantes sumamente resistentes al PAS en la población se ve disminuída por el pase repetido de la cepa PAS-resistente a medios PAS-privos y que es relativamente importante la tendencia a virar la mutación hacia la susceptibilidad. También se ha sugerido que los alelomorfos múltiples guardan relación con la PAS-resistencia.

Se ha observado el efecto combinado *in vitro* del PAS con respecto a realzar la acción tuberculostática de la dihidroestreptomicina o la isoniacida contra la cepa PAS-resistente, y se discute el mecanismo de este efecto. A la luz de estos resultados, parece que, aun cuando existe PAS-resistencia, la administración clínica de PAS combinado con dihidroestreptomicina o isoniacida es más eficaz que la de dihidroestreptomicina o isoniacida solas. Sugiérese, por lo tanto, que no se interrumpa la administración clínica de PAS debido a la aparición de PAS-resistencia, sino que continúe la administración de PAS combinado con estreptomicina o isoniacida, sobre todo estreptomicina, a menos que aparezca doble resistencia.

Resume

Les propriétés du Mycobacterium tuberculosis var. hominis (PAS-résistant)

Les propriétés du *M. tuberculosis* var. *hominis* (souche Aoyama B) résistant à l'acide para-aminosalicylique (PAS) ont été étudiées. Les conclusions sont les suivantes:

La population de la souche PAS-résistante est significativement hétérogène et une réduction du degré de la résistance au PAS a été observée dans un certain nombre de cellules individuelles de cette population. Même lorsque la résistance des cellules individuelles décroît du point de vue phénotypique, le génotype de la résistance au PAS, c'est-à-dire la propriété héréditaire produisant un taux de mutation augmenté au niveau élevé de la résistance au PAS, est conservée.

Il existe *in vitro* une action inhibitrice du PAS sur le développement de toutes les cellules individuelles de la souche PAS-résistante et cette action n'était considérée significative que lorsque le nombre de cellules viables inoculées était relativement bas.

Il a été suggéré que, dans cette population, la proportion des mutants présentant une résistance élevée au PAS est réduite par le transfert répété de la souche PAS-résistante dans des milieux dépourvus de PAS et que la tendance vers une mutation inverse, la re-

version à la sensibilité, est relativement significative. Il a été également suggéré que de multiples gènes allèliques sont intéressés dans la résistance au PAS. *In vitro* l'effet synergique du PAS, en renforçant l'action de la dihydrostreptomycine ou de l'isoniazide sur la souche PAS-résistante a été observée et le mécanisme de cette action a été commenté. D'après ces résultats, il semblerait que même en présence d'une résistance au PAS, l'administration clinique de PAS en association avec la dihydrostreptomycine ou l'isoniazide est plus efficace que l'administration exclusive de la dihydrostreptomycine ou de l'isoniazide. En conséquence, l'auteur suggère de ne pas interrompre l'administration clinique du PAS à cause de l'apparition d'une résistance au PAS, mais il préconise de poursuivre l'administration de PAS en association avec la streptomycine ou l'isoniazide, spécialement avec la streptomycine, à moins que la double résistance ne se manifeste.

Acknowledgment

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SEX DIFFERENCES OF NORMAL AND IMMUNIZED MICE IN RESISTANCE TO EXPERIMENTAL TUBERCULOSIS[1,2]

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INTRODUCTION

Sex differences in resistance to tuberculosis have long been known to exist among humans, females being more susceptible through some of their sexually active years. Lurie (1) and others have studied the effects of administration of various hormones on resistance to experimental tuberculosis. A naturally occurring sex difference in resistance to experimental tuberculosis was noted in immunization experiments on young Olitsky white mice and is the subject of the present report. Unimmunized females were slightly more susceptible than unimmunized males, and this difference was greatly increased by immunization, using BCG with or without *n*-hexadecane as an adjuvant. The immunologic aspects of these experiments are reported in the subsequent paper in this series (2).

MATERIALS AND METHODS

Olitsky "enteritidis-resistant" white mice were raised from a colony which has been maintained by the School of Medicine of the University of Southern California for more than ten years. Sexes were separated shortly after weaning and, with few exceptions, all females were virgins. All mice were sexually mature, being from six to ten weeks old on entering the experiments, and the ages of all mice that formed the groups in each experiment were kept as comparable as possible. Groups within the experiments comprised 20 or more mice of both sexes, and the numbers of males and females within each group generally approximated one another. The distribution of ages in the two sexes was unbiased.

Details of BCG vaccine preparation, doses, et cetera, are reported in the subsequent paper (2). Vaccines were generally injected by the intraperitoneal route but also subcutaneously, intramuscularly, or intravenously. The vaccines were either live or heat killed at 70° C. for an hour and were administered with and without the addition of *n*-hexadecane. All protocols included untreated control mice and in many experiments "hexadecane only" controls were also used.

The mice were challenged intravenously with 1 mg. wet weight of H37Rv from ten days to three weeks after vaccination. They were observed daily and autopsies were performed after death or at the end of the experiments. Less than 2 per cent of all animals died of intercurrent or accidental causes, and these were excluded from consideration. Experiments were generally terminated between five and eight weeks after challenge.

Statistical study of the distributions of fatality times showed, as expected (3), that they were markedly skewed on an arithmetic scale but that they were normally distributed on an exponential scale. All computations of fatality times therefore utilized the logarithms of

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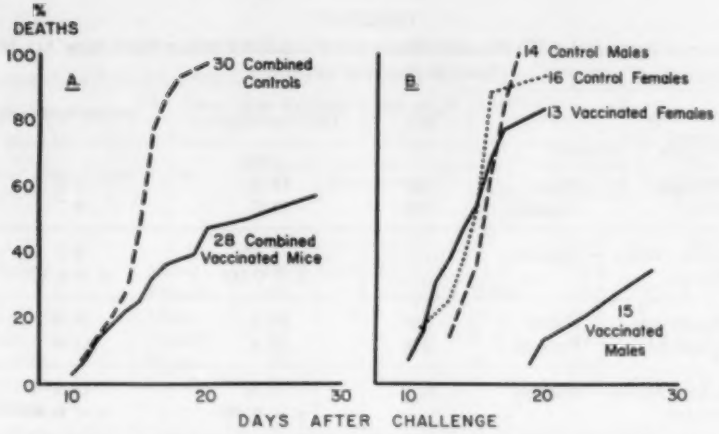

FIG. 1*A* and *B*. Cumulative mouse death percentages from one experiment.

the days from challenge to death. The antilog of the mean log yielded the geometric mean (n^{th} root of the product of n terms). Tests of significance of difference between means were carried out in the usual way: $t = (\bar{x}_1 - \bar{x}_2)/\sqrt{V_{\bar{x}_1} + V_{\bar{x}_2}}$, where $\bar{x}_1$ and $\bar{x}_2$ are the means, $V_{\bar{x}_1}$ and $V_{\bar{x}_2}$ are variances of these means, and x_i is $\log_{10}$ of fatality time. Tests of significance of the difference between two death rates utilized the expression $t = (p_1 - p_2) \Big/ \sqrt{\frac{p_1 q_1}{n_1} + \frac{p_2 q_2}{n_2}}$, where p is the proportion of deaths; q, of survivals; $p + q = 1$; and n is the number inoculated.

RESULTS

In tests on immunization of mice with live and heat-killed BCG, given with and without hexadecane and by various routes, 20 control groups and 28 immunized groups were available for a study of differences in resistance of males and females to H37Rv. It is emphasized that all groups contained males and females which (with few exceptions) were present in approximately equal numbers. Hence, after making sex resistance comparisons within each individual group, it was permissible to pool the male *versus* female comparisons of appropriate groups. Before showing the summation of these data, however, the results of part of one typical experiment may be reviewed.

Fifteen male and 13 female mice were vaccinated intraperitoneally with 0.2 mg. of heat-killed (70°C. for one hour) BCG in 0.25 ml. 1 per cent hexadecane-in-water emulsion twenty days before challenge with H37Rv. There were 14 male and 16 female untreated control mice. Death percentages at different days of the combined males and females within each group are charted in figure 1 (*A*), but this gives a false picture. What actually occurred is shown in figure 1 (*B*) when the males and females within each group are separated. The curves for fatalities among the control mice and the vaccinated females were approximately the same. In this test the vaccinated females failed to show an enhanced resistance, but the

TABLE 1

SUMMARY OF RESISTANCE DIFFERENCES BETWEEN MALE AND FEMALE MICE FROM ALL BCG VACCINATION EXPERIMENTS

Combined Groups	Sex	Number of Mice	Geometric Mean Death Times After Challenge	Survival Percentages
			days	
Control mice	Males	256	15.8	5.9
	Females	245	14.7	5.7
Difference: Males − females			1.1*	0.2
Significance			$p < 0.001$	$p = 0.9$
Specifically immunized mice	Males	350	18.9	26.9
	Females	356	16.3	13.8
Difference: Males − females			2.6†	13.1
Significance			$p < 0.001$	$p < 0.001$

* The mean log fatality time of males was 1.1976 and of females 1.1680, the difference being 0.0296. The standard error of the difference between the two means was 0.0071, yielding a t value of 4.17 for 499 degrees of freedom. The p value is <0.001.

† The difference between sexes in the immunized group is signicantly greater than the difference between sexes in the control group ($p < 0.01$).

vaccinated males developed a marked immunity. Two-thirds of them survived and the remainder died late in the experiment.

The females in many other experiments did not respond so poorly to immunization as those in figure 1 (*A* and *B*), but they generally fell below the males in their response to vaccination. As already stated, there were many groups of BCG-vaccinated mice and controls which could be examined for differences in resistance of males and females to experimental tuberculosis. Males and females were included in each experimental group and were generally present in approximately equal numbers. Males and females within each vaccinated group had been immunized by identical methods. Thus, a general summation of results for all immunized mice is possible, even though different immunization techniques were used in the various experiments with differing degrees of success (2). The controls of all experiments are similarly combined. They include (*a*) untreated controls used in each experiment, and (*b*) additional controls treated with hexadecane only at the time of vaccination, which were employed in some protocols (2). These summations are presented in table 1.

As may be seen in table 1, male control mice were slightly but significantly more resistant to experimental tuberculosis than the females, when evaluated by death time differences. This is not shown by the less sensitive method of comparing survival percentages. Immunization significantly increased this difference which is now demonstrated by both methods of evaluation. Males appear to develop more immunity than females when immunized by identical means (6).[2]

[2] Dr. Hubert Bloch reports that he has noted analogous sex differences in vaccinated CF_1 colored and C57 black mice, but not in unvaccinated controls.

TABLE 2

DIFFERENCE IN RESISTANCE OF MALES AND FEMALES

Summary of Five Experiments, in Each of Which Heat-Killed BCG Was Used Alone and Also With *n*-Hexadecane as an Adjuvant

Combined Mouse Groups	Sex	Number of Mice	Survival Percentages	Survival Percentage Differences Males—Females	Geometric Mean Death Times After Challenge	Geometric Mean Death Time Differences Males — Females
					days	*days*
Controls	Males	136	6.6	1.8	15.9	0.6
	Females	124	4.8	($p > 0.50$)	15.3	($p < 0.10$)
BCG (70°C.–1 hour) only	Males	69	21.7	16.9	19.8	3.6
	Females	63	4.8	($p < 0.01$)	16.2	($p < 0.001$)
BCG (70°C.–1 hour) plus hexadecane	Males	70	54.3	28.7	23.1	4.4
	Females	78	25.6	($p < 0.001$)	18.7	($p < 0.01$)

In five successive experiments, 0.2 mg. of heat-killed (70° C. for one hour) BCG was injected intraperitoneally both in aqueous suspension and mixed with small amounts of *n*-hexadecane. These experiments are combined in table 2.

As may be seen in table 2, there is a small, but not statistically significant, increase in resistance of the unimmunized males over the corresponding females. This difference increased considerably and became significant when the mice were immunized. BCG alone, in comparison with controls, markedly increased the length of life and the percentage of the males which survived, but in females there was no change in survival rate and only a slight ($p = 0.05$) increase in length of life. When hexadecane was used as an adjuvant, an additional degree of resistance was shown by both sexes; the males again responded much more than the females to this immunization.

DISCUSSION

Obviously, it would be ideal to establish the fundamental statistical relations between challenge doses of H37Rv and the response of mice and, with this information, make quantitative estimates of resistance. Without such information, only qualitative deductions can be drawn from the data available.

Unimmunized males were slightly more resistant to experimental tuberculosis than unimmunized females. This small difference might easily have been missed had there not been numerous experiments in which it almost invariably appeared. Other workers (4, 5) have actually tabulated small resistance differences between unimmunized male and female mice, but these differences were not considered significant and do not seem to have been investigated further.

The slight resistance differences between unimmunized male and female mice here reported became more obvious in specifically immunized animals. The differences also increased as the degree of specific immunity was raised. The males responded better to immunization than did the females.

The question may be raised of whether the small sex difference in resistance of

unimmunized mice is due to a greater reactivity of the specific immune mechanism in males. The H37Rv challenge dose acts in at least two ways. It infects and it also immunizes. Its immunizing action must play a minor part in previously unimmunized controls, for here it acts mainly as a primary antigenic stimulus, and the processes of infection run far ahead of immunization. Still immunization must play a part in the final outcome for controls. A few controls survived; some of these survivors were bled and specific agglutinins against *M. tuberculosis* were demonstrated in their sera.

In the case of previously vaccinated mice, the immunizing action of the challenge must be increased; for it now acts as a specific booster to an immune level already established by vaccination. Thus, if males are more responsive to specific immunization than females, the combination of vaccination plus challenge should accentuate the difference between males and females beyond that observed in controls. The hypothesis that the difference in resistance of the two sexes is the result of a difference in their ability to respond to specific immunization is consistent with the experimental data presented.

It is well known that the morbidity and mortality statistics of tuberculosis in the United States show a frank elevation for females, beginning at about the age of puberty and continuing throughout a fairly large part of their child-bearing period. The data presented in this communication re-enforce the possibility (1) that this increased susceptibility of women during their sexually active years may possibly reflect an endocrine influence upon responsiveness of the specific immune mechanism. Further studies on experimental animals to investigate the endocrine aspects of resistance to tuberculosis appear warranted. In addition, a study of the hormonal patterns of women with tuberculosis and their tuberculin-positive and -negative controls would seem justified. Certainly, any factor which may significantly increase the incidence and death rate of human tuberculosis warrants careful study.

Summary

Unimmunized, sexually mature, young male mice showed more resistance to experimental tuberculosis than did corresponding virgin females. This difference was slight but significant.

The sex difference in resistance of mice was significantly increased by immunization with BCG. This difference appears attributable to a difference in the responsiveness of the specific immune mechanism in the two sexes.

Sumario

Diferencias por Sexo de los Ratones Normales e Inmunizados en su Resistencia a la Tuberculosis Experimental

Los ratones machos jóvenes, maduros sexualmente, no inmunizados revelaron más resistencia a la tuberculosis experimental que las correspondientes hembras virgenes. Esta diferencia fué ligera, pero significativa.

La diferencia por sexo en la resistencia de los ratones fué realzada significativamente por la inoculación con BCG. Esta diferencia parece ser imputable a una diferencia en la sensibilidad del mecanismo inmune específico en los dos sexos.

Resume

Les différences de sexe chez la souris normale ou immunisée dans la résistance à la tuberculose expérimentale

Les jeunes souris mâles non immunisées ayant atteint la maturité sexuelle offrent une plus grande résistance à la tuberculose expérimentale que les femelles vierges correspondantes. Cette différence est légère mais significative.

Chez la souris la différence de sexe à l'égard de la résistance était notablement augmentée par l'immunisation au BCG. Cette différence paraît imputable à une différence dans la réactivité du mécanisme immun spécifique dans les deux sexes.

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THE ADJUVANT EFFECT OF N-HEXADECANE IN MOUSE VACCINATION TESTS WITH HEAT-KILLED BCG[1,2,3]

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Introduction

Active immunization against tuberculosis is being increasingly evaluated by mouse vaccination tests. BCG and other live mycobacteria have engendered reasonably high immune levels, and killed bacteria or fractions thereof, both alone and particularly when suspended in adjuvants, have shown considerable promise (1–6). Many workers have contributed to this field, and the literature cited is far from complete. Mineral oils and waxy materials have received special attention as antigenic adjuvants, but they are variable mixtures of uncertain composition and may contain harmful components.

The present paper concerns mouse vaccination experiments on the adjuvant action of a mineral oil constituent, *n*-hexadecane, in combination with heated (70° C. for one hour) culturally dead BCG. The idea of vaccination with killed mycobacteria, provided that such vaccines can be made sufficiently effective, is of particular interest to the present writers. Killed vaccines should be more effective when mixed with proper adjuvants. Hexadecane was chosen as a trial adjuvant because of its known chemical composition, physical characteristics, low toxicity, and moderate tissue-irritating properties.

As vaccination experiments progressed, it became evident that the mice showed an unexpected sex difference in resistance to experimental tuberculosis, which is reported in the preceding paper (7). This sex resistance difference made it necessary to review all data from the vaccination experiments so that male vaccinated mice would only be compared with male controls, et cetera, and the same evaluation was necessary for females.

Materials and Methods

n-Hexadecane (Eastman practical) is an inert, slightly oily, colorless liquid which induces a low-grade peritonitis on intraabdominal injection into mice. It is absorbed slowly and elicits an abundant monocytic response. It forms a fine though not permanent emulsion when homogenized in water, and such a 1 per cent emulsion is well tolerated when injected intravenously in 0.25 ml. volumes. Much larger amounts can be safely administered by other routes.

A Rosenthal strain of BCG was employed for vaccines. After approximately three weeks' growth on Löwenstein-Jensen slants, it was heated on the slants in a water bath at 70°C.

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[2] Presented before the Medical Session, as part of *Section 1B*, at the annual meeting of the National Tuberculosis Association, New York, New York, May 21, 1956.

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for an hour and was culturally dead. It was then removed and weighed without further drying and was freshly suspended in distilled water for each experiment. *n*-Hexadecane was added and suspended by hard shaking after vaccine suspensions had been made.

The mice employed are described in the preceding paper (7). Experimental groups contained 20 or more animals divided about equally between the two sexes.

Mice were challenged intravenously with 1 mg. H37Rv from thirteen days to three weeks after vaccination. These organisms were carried on Löwenstein-Jensen slants, weighed without further drying, and suspended in distilled water only to avoid possible immunologic complications from such substances as Tween® albumin. The resultant somewhat clumpy suspensions (8) were injected in 0.25 ml. volumes. Injection orders were staggered so that no one whole group was inoculated early and another late in the course of challenge.

Daily observations were made after challenge. All mice were eventually examined at autopsy, either after death in an experiment or as survivors when the test was terminated. A few mice that died from causes other than tuberculosis were omitted from the protocols. The experiments were analyzed statistically by the methods employed in the preceding report (7).

Results

Five separate experiments on intraperitoneal vaccination with heated (70°C. for one hour) BCG have been run and are reported here. One 0.2 mg. dose of BCG was used throughout and, in each experiment, the immunity resulting from BCG alone was compared with the immune level engendered by BCG in a hexadecane emulsion. The amounts of hexadecane employed varied from 0.25 ml. of a 1 per cent to 1 ml. of a 2.5 per cent suspension.

Each experiment included a group of untreated controls and two tests also contained a total of five mouse groups which were treated with hexadecane only. Although these "hexadecane only" groups received doses spread over the 0.25 ml. of 0.1 per cent to 1 ml. of 2.5 per cent range, their response to challenge with H37Rv did not vary significantly from that of the untreated controls. Both categories were, therefore, classed as controls.

The experiments were terminated at 53, 50, 44, 35, and 28 days after challenge. All pointed in the same direction, and their results are combined in table 1.

It is indicated in table 1 that male mice which were vaccinated with heated BCG alone were significantly more resistant to challenge than male controls. The same comparison for females fails to show a difference by survival percentages, and female vaccinated mice were only just significantly more resistant than their controls when evaluated by the more sensitive death time comparison method. Mice of each sex, when immunized with heated BCG plus hexadecane, show significantly more resistance than was afforded by vaccination with heated BCG only.

Intraperitoneal injection of *n*-hexadecane, with or without BCG, followed by intravenous challenge with H37Rv resulted in the formation of small subserosal and (especially) intramesenteric abscesses in many mice. These "mesenteric" abscesses, initiating outside the peritoneal serosa, rarely caused adhesions. Their frequency was a function of the amount of hexadecane injected but bore no relation to the administration of BCG, which is illustrated in table 2.

Table 2 is compiled from one of the experiments summarized above. Untreated controls were compared with mice that received three different doses of hexa-

TABLE 1

FIVE EXPERIMENTS COMBINED. HEATED BCG INJECTED INTRAPERITONEALLY BOTH WITH AND WITHOUT HEXADECANE

Group Treatment	Sex	Number of Mice	Percentage of Survivors	Geometric Mean Death Times After Challenge
				days
Group A controls, untreated or treated with hexadecane only	Males	136	6.6	15.9
	Females	124	4.8	15.3
Group B BCG (70°C.–1 hour) only	Males	69	21.7	19.8
	Females	63	4.8	16.2
Group C BCG (70°C.–1 hour) plus hexadecane	Males	70	54.3	23.1
	Females	78	25.6	18.7

Intergroup Comparisons by			
Survival Percentages		Geometric Mean Death Times	
Males	Females	Males	Females
$B > A$ ($p < 0.01$) $C > B$ ($p < 0.01$)	$B = A$ $C > B$ ($p < 0.01$)	$B > A$ ($p < 0.001$) $C > B$ ($p = 0.02$)	$B > A$ ($p = 0.05$) $C > B$ ($p < 0.01$)

TABLE 2

DEVELOPMENT OF MESENTERIC ABSCESSES FOLLOWING INTRAPERITONEAL HEXADECANE

Group Number	Treatment of Mouse Groups		Percentage of Mice with Mesenteric Abscesses
	Hexadecane	BCG	
A	None	None	0
B	None	0.2 mg.	0
C	0.25 ml. of 0.1%	0.2 mg.	0
D	0.25 ml. of 1%	0.2 mg.	88
E	1 ml. of 2.5%	0.2 mg.	100
F	0.25 ml. of 0.1%	None	11
G	0.25 ml. of 1%	None	81
H	1 ml. of 2.5%	None	100

decane only, with groups that received heated BCG plus like amounts of hexadecane, and with mice given heated BCG only. The smallest hexadecane dose, 0.25 ml. of 0.1 per cent (*Group C*), failed to produce abscesses in the presence of BCG, and this same amount without BCG (*Group F*) caused only a minor proportion of abscess formation. The two higher doses of hexadecane, with and without BCG, caused high percentages of mice to develop mesenteric abscesses, while no abscesses were attributable to BCG alone. The abscesses contained many acid-fast rods. Hexadecane-induced inflammation probably trapped some circulating H37Rv challenge organisms, which then multiplied locally, with a resultant development of abscesses.

A minimal amount of hexadecane seemed necessary for satisfactory adjuvant action. Vaccination with BCG plus 0.25 ml. of 0.1 per cent hexadecane (table 2,

Group C) produced an immune level not significantly different from that caused by BCG alone (*Group B*). The *Group C* mice were omitted from the summary of table 1 as they could not legitimately be called "BCG only mice" and they had obviously received too little hexadecane to cause an observable adjuvant effect. The two higher hexadecane doses, however, enhanced the immunizing effect of BCG to the same extent and are part of table 1.

Discussion

Nonspecific tissue stimulation can result in enhanced resistance against many infections, as shown by Gay and associates (9) and others. Nyka (10) recently increased the resistance of mice against tuberculosis by injections of *B. abortus*, postulated that the immunity might be cellular, and did not believe that a common antigen to *B. abortus* and *M. tuberculosis* could play a part.

It was hoped that hexadecane alone might properly stimulate (or irritate) the tissue of mice and thus produce a nonspecific increase in resistance against tuberculosis. This is not a closed subject and more work on this nonspecific aspect of immunization is in progress. If proper administration of hexadecane alone is shown to confer increased nonspecific immunity, the question will still arise of whether the term "nonspecific immunity" is correct. Hexadecane might actually enhance the immunizing action of the challenge organisms *in vivo* (7).

Hexadecane showed definite adjuvant properties when mixed with heat-killed (70° C. for one hour) BCG and administered intraperitoneally to mice. The reasons for this adjuvant action are not completely understood. Hexadecane probably delays absorption of vaccine from the peritoneal cavity. Its mild irritating action may also help in conjunction with specific immunization. The small mesenteric abscesses which form in many mice that have received adequate amounts of intraperitoneal hexadecane plus heated BCG, followed by intravenous challenge with H37Rv, may actually amount to added antigenic depots of challenge organisms. Thus they could further the immunity already initiated by vaccination and later boosted by challenge (7). Work in progress on subcutaneous immunization of mice has demonstrated, however, that hexadecane may show a definite adjuvant action without the formation of abscesses.

Whereas adjuvants such as mineral oils are unknown mixtures, chemically defined adjuvants like hexadecane can be purified and carefully tested for toxicity and possible carcinogenic action, as well as for immunity-aiding properties. It is believed that such work should be extended.

Adjuvants can definitely enhance the immunizing potency of killed organisms. There is evidence that some killed strains of mycobacteria are more antigenic than killed BCG (1). Killed vaccines (bacterins) have certain advantages over live vaccines. They should be safer and could be shipped and stored more effectively than live vaccines. Virulent organisms of highest antigenic potency could be used as vaccines, *provided that they were truly killed*. Hence, killed mycobacteria plus proper adjuvants might well prove to be safe, effective, and practical vaccines.

Summary

The adjuvant properties of *n*-hexadecane were assayed by BCG vaccination experiments in mice.

A single intraperitoneal injection of heat-killed (70° C. for one hour) BCG resulted in significant immunity, especially in males, as indicated by a reduced fatality rate and/or increased survival time in relation to unvaccinated controls.

An admixture of hexadecane with the antigen increased significantly the degree of immunity, while hexadecane alone was without effect.

Sumario

El Efecto Coadyuvante de la n-Hexadecana en los Ensayos de Vacunación Murina con BCG Matado al Calor

Las propiedades coadyuvantes de la *n*-hexadecana fueron valoradas con experimentos de vacunación BCG en ratones.

Una sola inyección intraperitoneal de BCG matado al calor (70° C por una hora) dió por resultado inmunidad importante, sobre todo en los machos, según indicaron la reducida tasa de mortalidad y/o el mayor tiempo de sobrevivencia en relación con los testigos no vacunados.

Una mezcla de hexadecana con el antígeno acrecentó notablemente la potencia de la inmunidad, en tanto que la hexadecana sola no surtió efecto.

Resume

L'effet adjuvant de n-Hexadecane dans les tests de vaccination de la souris par le BCG tué par la chaleur

Les propriétés adjuvantes de *n*-hexadecane ont été déterminées par la vaccination expérimentale de la souris par le BCG.

Une seule injection intrapéritonéale de BCG tué par la chaleur (70°C pendant une heure) a donné une immunité de valeur significative, en particulier chez les mâles, comme le démontre la réduction du taux de mortalité ou l'élévation du temps de survie, ou les deux, par rapport aux sujets-témoins non vaccinés.

Un mélange d'hexadecane avec l'antigène augmentait de façon significative le degré de l'immunité, alors que l'hexadecane seul était sans effet.

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TUBERCULOSTATIC EFFECT OF CALF LUNG FATTY ACIDS ON HUMAN AND BOVINE TUBERCLE BACILLI[1,2]

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(Received for publication May 21, 1956)

INTRODUCTION

Rabbits and cattle possess a rather high degree of resistance to tubercle bacilli of human origin and yet are extremely susceptible to bacilli of bovine origin. It is tempting to postulate that this pattern of resistance is simply a manifestation of physiologic conditions in the host which make it a more unfavorable environment for multiplication and/or survival of human bacilli. In a previous publication from this laboratory it was shown that fatty acids derived from rabbit lung were more bacteriostatic *in vitro* for human than for bovine tubercle bacilli (1). Various fractionation procedures failed to reveal the identity of the active component (or components); however, it was believed that this might have been due to technical limitations of the methods used. Moreover, these studies were handicapped by the rather inadequate supply of lung tissue available for analysis.

The purpose of the present report is to describe investigations of fatty acids from calf lungs. As crude, unfractionated mixtures these fatty acids were also found to be more toxic for human than for bovine tubercle bacilli. An active component has been identified as palmitic acid.

MATERIALS AND METHODS

The source and maintenance of the strains of human (H37Rv) and bovine (Ravenel) tubercle bacilli used in these studies have been described elsewhere (1).

Fresh calf lungs were obtained from a slaughter house. They were washed free of superficial blood with distilled water, frozen at −20°C., and thawed just prior to use. Details of the fractionation procedures are presented below.

Fatty acids were determined according to the oxidative dichromate method of Bragdon (2). Saponification equivalents were determined by the method of Ketchum (3); and microdetermination of iodine numbers as described by Yasuda (4).

In vitro studies of the growth response of the human and bovine tubercle bacilli in the presence of the fatty acid fractions were performed in a modified Proskauer and Beck synthetic liquid medium as described previously (1).

OBSERVATIONS

For the isolation of free fatty acids, 100 gm. of calf lung were minced in a Waring Blendor with 500 ml. of a 3:1 mixture of ethyl alcohol and ether and placed in a 60°C. water bath for two hours. The alcohol-ether extract was decanted through Whatman No. 1 filter paper into a suction flask. A fresh sample of 3:1 alcohol-ether mixture was added to the residue

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[2] Presented before the Medical Session, as part of *Section 1B*, at the annual meeting of the National Tuberculosis Association, New York, New York, May 21, 1956.

TABLE 1

GROWTH RESPONSE OF HUMAN (H37Rv) AND BOVINE (RAVENEL) TUBERCLE BACILLI IN THE PRESENCE OF FREE FATTY ACIDS FROM CALF LUNG

Strain	Final Concentration of Fatty Acid	Amount of Growth-Days of Incubation			
		7	14	21	28
	(γ per ml.)				
H37Rv	5.00	−	−	−	−
	2.50	−	−	−	−
	1.25	++	+++	++++	++++
	Control	++	+++	++++	++++
Ravenel	5.00	−	−	−	−
	2.50	++	+++	++++	++++
	1.25	++	+++	++++	++++
	Control	++	+++	++++	++++

and it was re-extracted for two hours at 60°C. This second extract was poured through the same filter paper. The pooled filtrates were concentrated to near dryness under a vacuum and a stream of carbon dioxide, and the lipids were taken up in 200 ml. of ether. The ether solution was shaken with 100 ml. of 2 per cent sodium hydroxide. The alkaline solution was extracted twice with 100 ml. of ether, acidified with 20 ml. of 5 N hydrochloric acid, and re-extracted twice with 100 ml. of ether. The pooled ether extracts were re-saponified and acidified as described above, and the final ether solution of fatty acids was washed three times with 100 ml. portions of distilled water. The yield consisted of 175 mg. of mixed fatty acids having an iodine number of 86.

The results of growth studies *in vitro* (table 1) show that the free fatty acids from calf lung were markedly tuberculostatic, but slightly more so for the human than for the bovine organisms. The difference here, however, was not as pronounced as that found with the fatty acids from rabbit lung (1).

Since the earlier studies with rabbit lung had shown that active fatty acids could be prepared from phospholipid, phospholipid fatty acids were isolated from calf lung according to techniques similar to those described by Goldman and associates (5). In addition, a sample (2.34 gm.) of the mixed fatty acids was taken up in 40 ml. of ether and placed in an ethylene glycol monomethyl ether (Fisher) bath maintained at −40°C. with dry ice. At the end of one hour the insoluble acids were separated by filtration. Whole fatty acids, acids insoluble at −40°C., and acids soluble at −40°C. were found to have iodine numbers of 94, 55, and 115, respectively. The growth response of the human and bovine tubercle bacilli in the presence of each of the fatty acid fractions was measured in the usual fashion.

The results presented in table 2 indicate a greater susceptibility of the human organisms to phospholipid fatty acids from calf lung. Moreover, the active component appeared to reside in the fraction insoluble in ether at −40°C. The iodine number of this fraction, 55, suggested a mixture of saturated and monoethylenic acids.

In an attempt to further characterize the active component, a fresh sample of phospholipid fatty acids from calf lung was separated into saturated and unsatu-

TABLE 2

GROWTH RESPONSE OF HUMAN (H37Rv) AND BOVINE (RAVENEL) TUBERCLE BACILLI IN THE PRESENCE OF PHOSPHOLIPID FATTY ACIDS FROM CALF LUNG

Final Concentration of Fatty Acid	Amount of Growth (4 Weeks)					
	Whole Fatty Acids		Fatty Acids Insoluble at −40°C.		Fatty Acids Soluble at −40°C.	
	H37Rv	Ravenel	H37Rv	Ravenel	H37Rv	Ravenel
(γ per ml.)						
40.0	−	−	−	−	−	−
20.0	−	−	+	++++	−	−
10.0	−	++	++++	++++	−	−
5.0	++++	++++	++++	++++	−	−
2.5	++++	++++	++++	++++	++++	++++
Controls	++++	++++	++++	++++	++++	++++

TABLE 3

GROWTH RESPONSE OF HUMAN (H37Rv) AND BOVINE (RAVENEL) TUBERCLE BACILLI IN THE PRESENCE OF PHOSPHOLIPID FATTY ACIDS FROM CALF LUNG

Final Concentration of Fatty Acid	Amount of Growth (4 Weeks)			
	Saturated Fatty Acids		Unsaturated Fatty Acids	
	H37Rv	Ravenel	H37Rv	Ravenel
(γ per ml.)				
160.0	−	−	−	−
80.0	−	+++	−	−
40.0	++	+++	−	−
20.0	+++	++++	−	−
10.0	++++	++++	−	−
5.0	++++	++++	+	+
2.5	++++	++++	++++	++++
Controls	++++	++++	++++	++++

rated fractions by the lead salt method of Sinclair (6). The saturated acids had an iodine number of 3.4; the unsaturated acids, of 139. These two fractions were tested *in vitro* against the H37Rv and Ravenel strains and the results are shown in table 3. It will be seen that the differential effect toward human and bovine bacilli appeared to rest exclusively with the saturated fatty acids.

According to Kaucher and associates (7), beef lung phospholipids are composed of the following, in per cent dry weight of tissue: lecithin, 3.6 per cent; cephalin, 3.9 per cent; and sphingomyelin, 2.3 per cent. Since sphingomyelin, by virtue of its ether insolubility, can be rather readily separated from lecithin and cephalin (8), further fractionation of the mixed phospholipids seemed worthy of investigation.

A 3.4 gm. sample of calf lung phospholipids was taken up in 100 ml. of ether and placed at 5°C. under nitrogen. The next day the solution was centrifuged at 3,000 r.p.m. for fifteen minutes and the supernate, containing the lecithin and cephalin, was decanted and saved.

TABLE 4

GROWTH RESPONSE OF HUMAN (H37Rv) AND BOVINE (RAVENEL) TUBERCLE BACILLI IN THE PRESENCE OF SATURATED PHOSPHOLIPID FATTY ACIDS FROM CALF LUNG

Final Concentration of Fatty Acid	Amount of Growth (4 Weeks)			
	Sphingomyelin Fatty Acids		Lecithin-Cephalin Fatty Acids	
	H37Rv	Ravenel	H37Rv	Ravenel
(γ per ml.)				
60.00	−	−	−	−
30.00	−	−	+	+
15.00	−	−	+	++
7.50	−	++	++	++
3.75	++++	++++	++++	++++
1.87	++++	++++	++++	++++
Controls	++++	++++	++++	++++

The fine, white precipitate of sphingomyelin was transferred to a Soxhlet apparatus and extracted with ether for six hours to remove any ether-soluble material. The residue in the thimble was removed with 80 ml. of a 9:1 petroleum ether-methanol solution, evaporated to dryness under nitrogen, and weighed (0.8 gm.). The lecithin and cephalin in ether solution were evaporated down to 20 ml. under nitrogen, precipitated with acetone, dried under nitrogen, and weighed (2.2 gm.).

The sphingomyelin and lecithin-cephalin fractions were each placed in 100 ml. of 2 M alcoholic potassium hydroxide and refluxed on a boiling water bath for four hours under a stream of nitrogen. Two hundred milliliters of distilled water were added, and unsaponifiable material was removed with three 100 ml. petroleum ether extractions. The aqueous alcohol layer was then acidified with 5 N hydrochloric acid, and the fatty acids were extracted with three 200 ml. portions of ethyl ether. The ether solutions of the fatty acids were finally washed three times with distilled water. Since beef lung sphingomyelin has been shown to contain only palmitic and lignoceric acids (9), separation of these acids was not attempted at this point. The lecithin-cephalin fatty acids were, however, separated into saturated and unsaturated components by the lead salt procedure described previously. In view of earlier findings, the unsaturated acids were not studied further. The saturated fatty acids from the lecithin and cephalin, together with the sphingomyelin acids, were each examined for its tuberculostatic effect *in vitro*.

From the results presented in table 4 it appears that only with the sphingomyelin fatty acids could the typical differential response of the human and bovine tubercle bacilli be demonstrated. As mentioned previously, beef lung sphingomyelin contains only palmitic and lignoceric acids (9). However, it should be pointed out that, by the techniques used for its preparation in the present study, the sphingomyelin fraction probably also contained an appreciable quantity of dipalmityl lecithin (10). It seemed of interest, then, to determine the effect of lignoceric and palmitic acids on the H37Rv and Ravenel strains.

A sample of pure lignoceric acid from beef tissue was generously supplied by Dr. S. J. Thannhauser of the Pratt Diagnostic Clinic, New England Center Hospital, Boston. Through the use of solubility characteristics reported in the literature (9), relatively pure palmitic acid was isolated in this laboratory from a sample of calf lung sphingomyelin.

TABLE 5

GROWTH RESPONSE OF HUMAN (H37Rv) AND BOVINE (RAVENEL) TUBERCLE BACILLI IN THE PRESENCE OF PALMITIC ACID FROM CALF LUNG

Strain	Final Concentration of Fatty Acid	Amount of Growth-Days of Incubation			
		7	14	21	28
	(γ per ml.)				
H37Rv	320	−	−	−	−
	160	−	−	−	−
	80	−	−	−	−
	40	−	−	+	++
	20	+	+++	+++	++++
	10	++	++++	++++	++++
	Control	++	++++	++++	++++
Ravenel	320	−	−	−	−
	160	−	−	+	+
	80	−	++	++	++
	40	++	+++	+++	+++
	20	++	+++	+++	++++
	10	++	++++	++++	++++
	Control	++	++++	++++	++++

An ether solution of sphingomyelin fatty acids, prepared as described above, was placed at 5°C. in order to remove any possible traces of unhydrolyzed sphingomyelin. On the next day, the solution was centrifuged at 5°C. in a refrigerated centrifuge. This and all subsequent centrifugations were done at 3,000 r.p.m. for fifteen minutes. The supernate was evaporated to dryness under nitrogen, taken up in 30 ml. of petroleum ether, placed at −10°C. overnight, and centrifuged at −10°C. The supernate was taken to dryness under nitrogen and weighed (125 mg.). The dried residue was then re-dissolved in 30 ml. of petroleum ether, placed at 5°C. for one hour, and centrifuged. A rather pure (melting point, 51°C.; saponification equivalent, 248) sample of palmitic acid weighing 62 mg. was obtained from the supernate by repeated crystallization at −20°C.

The lignoceric acid did not inhibit growth of either the human or bovine tubercle bacilli in the range of concentrations tested (0.31 to 160 γ per ml.). The palmitic acid, on the other hand, was bacteriostatic and exhibited a greater toxicity for the human than for the bovine bacilli (table 5). Essentially the same results were obtained with a sample of reagent-grade palmitic acid obtained from Fisher Scientific Company.

It could be suggested that the tuberculostatic effect of palmitic acid observed *in vitro* might be due, not to the acid per se, but to a derivative thereof which is peculiar to conditions under which the present tests were conducted.

To examine this possibility, 10 mg. of palmitic acid (Fisher) contained in 1 ml. of ethyl ether were added to 25 ml. of the modified Proskauer and Beck liquid medium (pH 6.6) and autoclaved for eight minutes at 15 pounds of pressure (121°C.). This is essentially the procedure which had been used in the preparation of the *in vitro* tests (1). After it had cooled to room temperature the medium was thoroughly extracted with ether, and 9.3 mg. of material were recovered. Of the recoverable acid, 90 per cent was free palmitic, as determined

by titration (3). The remaining 10 per cent consisted of a salt of palmitic acid which, on acidification with dilute hydrochloric acid, gave a strongly positive test for magnesium by the Kolthoff reaction (11).

Discussion

It appears generally accepted that free, unesterified fatty acids, including palmitic, are inhibitory for tubercle bacilli and a wide variety of other bacteria *in vitro* in the absence of detoxifying agents such as serum albumin (12). It is interesting that, in studies conducted with palmitic acid, the toxicity was found to depend not on the concentration of acid, but rather on the ratio of palmitate to cells. The same concentration which is oxidized rapidly by a large quantity of cells is toxic when the number of cells is reduced (13). When it is available as the sole source of carbon, palmitic acid supports the growth of only a rather large inoculum of virulent human tubercle bacilli (H37Rv) and only within a very narrow range of concentrations (14). It is probable that other factors, as will be discussed below, also affect the results of studies conducted *in vitro*.

In addition to its bacteriostatic effect *in vitro*, palmitic acid has been reported to have antituberculous properties *in vivo*. In the majority of cases in three series of guinea pigs infected with human tubercle bacilli, Long and Vorwald (15) concluded that the extent of tuberculosis was less in those animals fed palmitic acid than in the controls. Nègre and associates (16) administered a series of saturated fatty acids to tuberculous guinea pigs in a subcutaneous dosage of 0.2 to 0.5 ml. every two weeks. Of the six active fatty acids, palmitate had the most marked and regular effect in retarding the appearance of lesions.

Dubos (17) has postulated that certain fatty acids which are produced normally, either in the course of tissue metabolism or by autolytic changes following necrosis, might play a role in the evolution of tuberculous lesions since some of them occur *in vivo* in the range of concentrations which are tuberculostatic *in vitro*. The results obtained in the present study offer suggestive, though by no means conclusive, evidence that the course of tuberculosis in cattle might be determined by the tissue level of free palmitic acid available to the infecting organisms. However, these results must be interpreted with considerable caution in view of the reports in the literature which appear to be at variance with the present findings.

Dubos (17), for example, reported complete inhibition of both H37Rv and Ravenel with 0.001 M sodium palmitate in a semisynthetic, oleic-acid–albumin medium (pH 6.4). Seiji (18) concluded that palmitic, as free acid, incorporated in 4 per cent glycerin agar was without effect on both human and bovine tubercle bacilli, while sodium palmitate was more toxic for tubercle bacilli of bovine origin than for human tubercle bacilli. It would seem that for the whole range of naturally occurring fatty acids not only the composition of the culture medium (17) and the status of the particular fatty acid, whether free acid or salt (18), but also a variety of other factors will determine the outcome of comparative studies conducted *in vitro* with human and bovine tubercle bacilli.

Further investigations of this type must await more precise knowledge con-

cerning both the physical and chemical conditions prevailing in the normal host and the changes, both quantitative and qualitative, which might be produced by the invading organisms. It is the nature of the environment in which they find themselves that probably determines whether the bacilli survive or die.

Summary

An attempt has been made to determine the mechanism underlying the resistance of cattle to human tubercle bacilli and their susceptibility to bovine bacilli. Free fatty acids and phospholipid fatty acids prepared from calf lung by standard chemical procedures were found to be tuberculostatic *in vitro*, but slightly more so for human (H37Rv) than for bovine (Ravenel) bacilli. The results of further chemical studies of the phospholipid fatty acids suggested that palmitic acid was the active component.

Sumario

Efecto Tuberculostático de los Ácidos Grasos del Pulmón de Ternero sobre los Bacilos Tuberculosos Humanos y Bovinos

Tratóse de determinar el mecanismo subyacente de la resistencia del ganado vacuno a los bacilos tuberculosos humanos y su susceptibilidad a los bacilos bovinos. Los ácidos grasos libres y los ácidos grasos fosfolípidos preparados del pulmón de ternero por los procedimientos químicos aceptados se mostraron tuberculostáticos *in vitro*, pero ligeramente más para los bacilos humanos (H37Rv) que para los bovinos (Ravenel). Los resultados de nuevos estudios químicos de los ácidos grasos fosfolípidos sugirieron que el ácido palmítico era el componente activo.

Resume

Effet tuberculostatique d'acides gras du veau sur les bacilles tuberculeux humains et bovins

Il a été procédé à des tentatives de détermination du mécanisme conditionnant la résistance du bétail au bacille tuberculeux humain et sa sensibilité au bacille bovin. Les acides gras libres et ceux des phospholipides préparés à partir de poumons de veau par des techniques chimiques standard ont été trouvés bactériostatiques *in vitro*, et légèrement plus pour le bacille humain (H37Rv) que pour le bacille bovin (Ravenel). Les résultats d'études chimiques complémentaires sur les acides gras des phospholipides suggéraient que l'acide palmitique était le composant actif.

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Case Reports

EXTRAPLEURAL MESENCHYMOMA[1]

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The lack of any recorded instance of mesenchymoma occurring in an extrapleural location in the chest wall prompts the report of the following case.

Case Report

A 32-year-old white man was admitted to the Syracuse Veterans Administration Hospital on March 8, 1954, because of the incidental finding of an abnormal shadow on a chest roentgenogram obtained in the course of a routine examination.

The patient was asymptomatic and his past history was noncontributory. On physical examination he was a well-developed young man who did not appear to be ill. Blood pressure was 126 systolic 80 diastolic in mm. of mercury; pulse, 80 per minute, and his temperature was 99.2°F. No abnormal physical findings were present. Laboratory findings, including hematocrit, erythrocyte and differential counts, hemoglobin, erythrocyte sedimentation rate, urinalysis, serologic test for syphilis, and sputum culture for tubercle bacilli and other pathogens, were all normal. A routine chest roentgenogram (figure 1*A*) showed a homogeneous shadow, 9 by 6 cm., with a moderately well-defined border occupying the outer two-thirds of the lung field between anterior ribs one and three on the right. It was better defined on the lateral view (figure 1*B*) in which its location was posterior. A 4- by 5-inch film obtained nine years previously (1945) upon the patient's separation from the U.S. Army showed a similar shadow, but about one-fourth in size, in the same location.

On March 19, 1954, a thoracotomy was performed. A tumor mass (figure 2) with three lobulations was adherent to the chest wall between the third and sixth ribs posterolaterally in a subscapular position. There was no evidence of mediastinal lymph node or lung abnormalities, and no pleural adhesions were present. The parietal pleura overlying the mass was intact and, when a portion of this was stripped, it was evident that the tumor could not be cleanly separated from intercostal musculature. By a block resection of the chest wall, the entire tumor with an adequate free margin was resected. This created a 6- by 12-centimeter defect in the thorax which was for the most part subscapular.

The closure of the chest was accomplished by suturing the fascia of the subscapularis to the margins of the defect and then closing the chest in the usual manner.

Pathologic description: The surgical specimen (S54-249) consisted of a portion of chest wall comprised of segments of four ribs with adjacent soft tissues. On the pleural aspect, there was an irregular coarsely lobulated mass, overlaid by parietal pleura, that measured 8 by 5.5 by 1.5 cm. The pleural aspect was glistening and varied from purplish-gray to yellow, and the pleura was focally thickened up to 0.3 cm. The cut surfaces of the mass varied from gray mottled with pale gray, moderately firm, somewhat bosselated and bulging, on one end, to focally spongy, with irregular distortion by numerous blood-containing channels and focally discolored by reddish-purple and grayish-purple, on the other end. In no place did the mass invade the pleura, nor was there gross evidence of extension into the intercostal spaces.

Microscopic examination of seven sections revealed a somewhat poorly encapsulated

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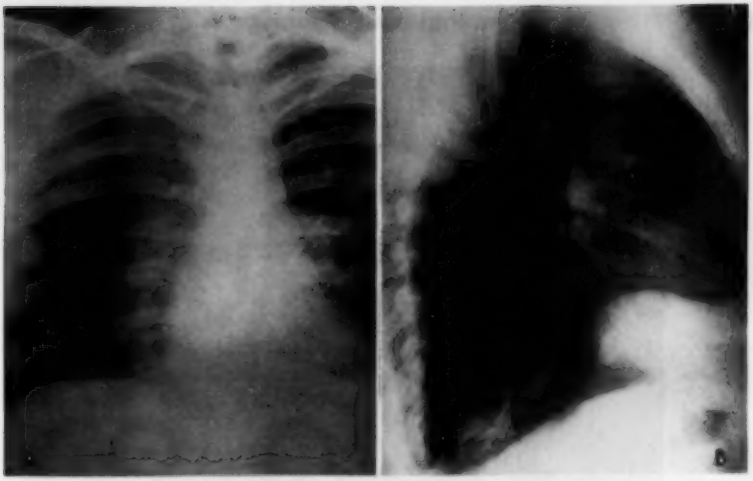

FIG. 1*A*. Postero-anterior chest roentgenogram obtained in January, 1954
FIG. 1*B*. Right lateral chest roentgenogram obtained in January, 1954

FIG. 2. Photograph of pathologic specimen. Note lobulate appearance with smooth pleural surface.

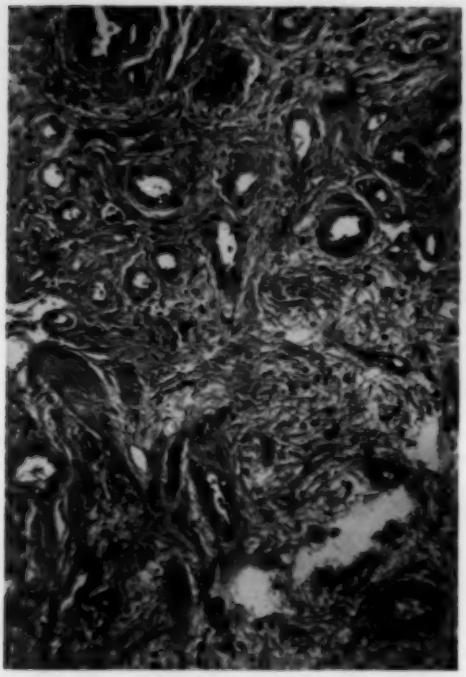

FIG. 3. Photomicrograph of pathologic specimen. Note the numerous vascular channels, individual smooth and striated muscle fibers, fibrous connective tissue bands, and small zones of mature fat, all embedded in a myxoid stroma.

mesodermal tumor comprised of multiple diversified elements, all of which were of mature type without any apparent organization of pattern. These elements included numerous vascular channels of both venous and arterial (arteriolar) types, individual scattered smooth and striated muscle fibers, fibrous connective tissue bands, and small zones of mature fat, all embedded in a pale blue myxoid stroma (figure 3). There was no evidence of atypism. The pathologic diagnosis was mesenchymoma, benign type, arising in extrapleural connective tissues. Dr. Arthur Purdy Stout, Professor of Surgical Pathology at the College of Physicians and Surgeons of Columbia University, examined the slides in this case and concurred with the above diagnosis. Dr. Stout commented, however, that the amount of myxoid tissue in this tumor was unusual and that one should be wary of recurrence. He further stated that he had seen numerous benign tumors of this sort, but none with this site of origin.

The postoperative course was uneventful and the patient was discharged from the hospital on April 6, 1954. On March 1, 1956, he was well, and physical examination was normal except for a small area of paradoxical motion in the interscapular area on the right. A chest roentgenogram (figure 4) at this time was normal except for the absence of the excised ribs. The results of pulmonary function studies performed on March 5 and 6, 1956, are shown in the table. The patient refused to allow bronchospirometry to be carried out.

FIG. 4. Postero-anterior chest roentgenogram obtained in March, 1956

COMMENT

Although the term *mesenchymoma* had been used for many years, Stout in a comprehensive discussion and review of the subject of mixed tumors in 1948 (1) suggested that its use be confined to a group of neoplasms in which two or more tissues of mesenchymal origin take part to form a single mass. He pointed out that both benign and malignant varieties of this tumor occur and emphasized that, although they have been reported more frequently in the urogenital and mammary systems, they are not confined to these regions. Thus, of his six original cases, four were in the lower extremity and one was in the neck. Other case reports have described these tumors in the liver (2), mesentery (3), retroperitoneal space (4), abdominal wall (5), low back region (6), between lobes of the lung (7), and in the anterior mediastinum (8).

The cases described by Symmers and Nangle (9) are of particular interest from the standpoint of treatment. Despite the fact that the tumor was histologically benign in three of the four patients presented by these writers, local recurrence took place in all after what was believed to be complete excision. Local invasiveness eventually led to the death of two of these patients. Symmers and Nangle point out that these neoplasms have a slow initial growth, recur locally at progressively shorter intervals after excision of apparently encapsulated masses, and that the recurrent tumors are more cellular and less well differentiated. In their experience the recurrent tumors were "moderately radiosensitive." Metastases have occurred in numerous instances (1, 5, 6, 8).

TABLE 1

RESULTS OF PULMONARY FUNCTION STUDY*

	Actual	Normal
Vital capacity (cc.)	4,127	4,216
Residual lung volume (cc.)	1,304	1,054
Total lung capacity (cc.)	5,431	5,270
Residual volume/total capacity × 100 (%)	24	20
Maximal ventilatory capacity (L. per min.)	127	151
Ventilation (L./min./m² BSA)		
Rest	4.28	3.2 ± 0.65
Exercise†	9.83	9.7 ± 0.81
First minute recovery	17.79	12.4 ± 2.40
Arterial oxygen saturation		
Rest	96	94–97
First minute recovery	96	94–97

* Lung volumes and ventilation at body temperature, ambient pressure, and saturated with water vapor at body temperature.

† Standard exercise = stepping up and down a platform 20 cm. high thirty times in one minute.

Since the tumor had been present nine years before admission in the case reported above, it may be presumed that its growth had probably been slow. In roentgenographic appearance, it was most like a pleural mesothelioma. Approximately two years after complete excision, no local or distant recurrence has been observed.

Studies of pulmonary function (table 1) made two years after operation were within normal limits except for a slight reduction of maximal ventilatory capacity and moderate hyperventilation. Apparently, the support afforded by the scapula over the decostalized portion of the chest wall was sufficient to prevent significant overdistention.

SUMMARY

Mesenchymomas, rare tumors containing two or more tissues of mesenchymal origin, may occur in the extrapleural tissues of the chest and simulate the roentgenographic appearance of a mesothelioma. As such tumors may be malignant, they should be completely excised.

SUMARIO

Mesenquimoma Extrapleural

Los mesenquimomas, tumores raros que contienen dos o más tejidos de origen mesenquimatoso, pueden presentarse en los tejidos extrapleurales del tórax y simular radiográficamente el aspecto de los mesoteliomas. Como pueden ser malignos, deben excindirse totalmente.

RESUME

Mésenchymome extrapleural

Les mésenchymomes, tumeurs rares renfermant deux ou plusieurs tissus d'origine mésenchymateuse, peuvent apparaître dans les tissus extrapleuraux thoraciques et simuler l'image radiologique d'un mésothéliome. Comme ces tumeurs sont malignes leur excision complète est nécessaire.

Acknowledgment

The writers wish to thank Mr. John Potrafka for his technical assistance in the study of pulmonary function.

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PULMONARY MALIGNANCY AND EOSINOPHILIA[1]

A Discussion and Case Report

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It is generally accepted that an eosinophil count greater than 6 per cent in peripheral blood is indicative of a pathologic state and is found in a wide variety of diseases (1, 2). However, eosinophilia in association with neoplastic diseases is considerably less common and has been only sporadically reported in the literature during the past sixty years. The primary site of such neoplasms has been located in widely scattered regions of the body. In some cases local tissue eosinophilia of high degree accompanied the neoplasms.

In the following instance an extreme eosinophilia in the peripheral blood and pleural fluid and histologic examinations of pleural tissue aspirated by a Vim-Silverman needle led to a presumptive diagnosis of eosinophilic granuloma of the pleura. However, the diagnosis of bronchogenic carcinoma with pleural metastases was confirmed by bronchoscopy and thoracotomy.

Case Report

G. P., a 49-year-old, divorced Philippine seaman, was admitted to the hospital on June 22, 1955, with a two months' history of dyspnea, cough, and expectoration which was occasionally blood streaked. There was no history of weight loss, fever, night sweats, or undue fatigue, and no history suggesting allergic difficulty or parasitic infestation. Approximately one month prior to admission, the patient noted the presence of acneiform lesions over the skin of the back and anterior chest wall, which soon disappeared, leaving an area of hyperpigmentation.

Physical examination revealed a well-developed, muscular, Philippine male in no acute distress. The vital signs were normal. Positive findings were confined to the chest where there was dulness to percussion over the left lower lateral and posterior sections, with some diminution of movement of the chest wall. A few moist rales were noted at the left apex anteriorly and posteriorly. No lymphadenopathy was noted.

Laboratory data: The hemogram revealed a sedimentation rate (Wintrobe) of 34 mm. per hour; hemoglobin, 13.7 gm.; hematocrit, 42 per cent; leukocyte count, 6,900 per cu. mm.; neutrophils, 48 per cent; lymphocytes, 28 per cent; eosinophils, 24 per cent. Repeated peripheral blood examinations revealed a leukocyte count varying between 8,000 and 11,000 per cu. mm. and eosinophilia varying from 12 to 27 per cent. Absolute eosinophil counts ranged from 1,200 to 1,700 per cu. mm. Urinalysis was normal and a VDRL flocculation test for syphilis was nonreactive. A skin test with purified protein derivative, first strength (0.0002 mg.), elicited a 2-plus reaction. The fasting blood sugar was 102 mg. per 100 ml.; blood-urea-nitrogen, 19 mg. per 100 ml.; the serum albumin, 3.63 gm. per 100 ml.; and the globulin, 2.97 gm. per 100 ml. A cold agglutination test was negative. The total cholesterol value was 183 mg. per 100 ml.; the alkaline phosphatase, 1.05 Bodansky units; and the inorganic phosphate, 3.45 mg. per 100 ml. Repeated examinations of the stool for ova and parasites were negative. Pleural fluid aspirated shortly after admission revealed a specific gravity of 1.018, a leukocyte count of 28,700 per cu. mm., with 82 per cent eosino-

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[2] Deceased.

phils. Cultures of sputum and pleural fluid for tubercle bacilli and fungi were negative. Cytologic examination of sputum revealed no evidence of neoplasm. Eosinophil cells were conspicuously absent in the sputum.

Hospital course: The peripheral blood and pleural fluid eosinophilia suggested the possibility of parasitic infestation. However, as noted above, stool examinations for parasites were negative. Examination of the bone marrow indicated only hyperplasia of the myeloid elements with eosinophilia. Roentgenographic studies of the entire skeletal system in an effort to uncover possible eosinophilic granulomas of bone were negative. Pleural biopsy from the left chest with the Vim-Silverman needle revealed a granulomatous inflammatory lesion with an abundance of eosinophils. This was considered by Dr. James Bennett, the hospital pathologist, to be histologically compatible with a diagnosis of "eosinophilic granuloma of pleura, with acute pleuritis."

Further examinations included bronchoscopy, which revealed a stenosis of the left lower lobe bronchus and obstruction by a thickened, irregular, reddened mucosa. Biopsy of this lesion revealed squamous cell carcinoma. A thoracotomy was performed on August 2, 1955. The parietal pleura contained extensive nodular subpleural infiltrates. A large mass was felt near the hilum. It was the surgeon's opinion that the lesion was inoperable, and after pleural biopsy the surgery was discontinued. The pleural biopsy exhibited metastatic squamous cell carcinoma with areas of necrosis and connective tissue reaction simulating eosinophilic granuloma.

Comment

Isaacson and Rapoport (3) state that Reinbach was the first to report a case of eosinophilia associated with malignancy in 1893. Since that time, 33 cases have been reported in the literature. Among the cases reported, the leukocyte count varied from 6,000 to 140,000, and the eosinophil count from 10 to 70 per cent (3–6).

It is interesting to note that more than 90 per cent of the reported cases have clinical or autopsy evidence of metastatic lesions (3, 4). The incidence of peripheral blood eosinophilia and malignancy has shown no predilection for sex, type of tumor, or any particular organ.

Kappis (7) was the first to report peripheral blood eosinophilia in association with bronchogenic carcinoma.

The extensive report of Isaacson and Rapoport (3) indicated that, of 2,363 cases of neoplastic disease, only 15 revealed peripheral blood eosinophilia, giving an incidence of 0.54 per cent. It is apparent then that the incidence of eosinophilia and malignant neoplastic disease is considerably higher than the small number of reported cases would lead us to believe.

It appears that local tissue eosinophilia is not uncommonly associated with malignant neoplastic disease (2, 3, 6, 8, 9). This was particularly true in the case reported herein in which there was not only marked local tissue eosinophilia associated with the bronchogenic carcinoma but also a rather intense degree of eosinophilia in the pleural fluid. It is, of course, impossible to state whether these eosinophil cells are of myeloid origin or whether they originate locally. At any rate, it is commonly believed that eosinophilia within the local tissue and the blood is due to an allergic response to broken-down protein from the malignancy.

A number of writers have suggested the following possible etiologies for the eosinophilia associated with malignant neoplastic disease.

> Previous writers have noted the presence of necrosis of tumor tissue in most of their cases. They postulated the theory that the products of protein breakdown cause the overproduction of eosinophils. This theory appears rather strongly supported by Isaacson and Rapoports's case, wherein the peripheral eosinophil count

returned to normal following gastrectomy for gastric cancer, but then again became elevated after the supervention of necrotic metastases. Weiss (8), however, attempted to reproduce this phenomenon by injecting necrotic tumor tissue from such a patient, but failed to produce an eosinophilic response.

Paviot (10), in 1935, suggested that the eosinophil cells had their origin in the connective tissue surrounding the tumor. In support of this theory is the observation that blood coming from the involved area has a higher eosinophil count than blood coming from the same organ of the other side. In the present case it had been hoped at operation to determine the eosinophil count of blood going to the left lower lobe (pulmonary artery to the left lower lobe) as compared with the eosinophilic content of blood coming from the same lobe (pulmonary vein from the left lower lobe). It had also been hoped to compare these figures with those obtained from blood going to and from the upper lobe. Unfortunately, however, the lesion was so advanced that these procedures could not be performed.

Pisa, referred to in (3), in 1931 believed that a familial eosinophilic predisposition was necessary to provoke an eosinophilic response in malignant tumors. It was thought that the foreign protein of the tumor cells was the provocative agent. No strong evidence has been marshalled in favor of this theory.

The possibility of eosinophilia's being due to a vagal reflex was presented by Strisower, as referred to by Isaacson and Rapoport (3). Strisower reported a case of hepatoma, which had metastasized to the cervical lymph nodes and had compressed the vagus nerve. The eosinophilia disappeared following removal of the nodes.

Querido (11) has suggested that the presence of eosinophilia might represent an aid to the differential diagnosis of malignancy. However, it would appear that the presence of eosinophilia may be a more useful guide to prognosis inasmuch as more than 90 per cent of patients with peripheral blood eosinophilia associated with malignancy exhibited evidence of metastatic spread. The present writers are inclined to agree with the view of Isaacson and Rapoport (3) that eosinophilia, when it occurs with malignant tumors and when other causes can be excluded, is indicative of metastatic dissemination and consequently indicates a poor prognosis.

Summary

A case of bronchogenic squamous cell carcinoma with pleural metastases associated with a pronounced eosinophilia of blood and pleural fluid is described in association with a review of the literature.

Sumario

Cáncer Pulmonar y Eosinofilia: Discusión y Presentación de Un Caso

Descríbese un caso de carcinoma escamocelular broncógeno con metástasis pleurales, asociado con acentuada eosinofilia de la sangre y del líquido pleural. Repásase la literatura y se discuten los factores etiológicos y la importancia de la eosinofilia observada en asociación con neoplasias malignas.

Resume

Cancer pulmonaire et éosinophilie: Commentaires et observation d'un cas

Description d'un cas d'épithélioma pavimenteux bronchogène avec métastases pleurales accompagné d'une éosinophilie élevée dans le sang et le liquide pleural. La littérature est analysée et les facteurs étiologiques ainsi que la signification de l'éosinophilie associée aux tumeurs malignes sont commentés.

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Editorial

Changes Ahead for the American Trudeau Society?

The American Trudeau Society has an extraordinary heritage from the past in its record of achievements in the control, the prevention, and the care of tuberculosis. Its leadership in education research in thoracic disease, and in the publication of THE AMERICAN REVIEW OF TUBERCULOSIS AND PULMONARY DISEASES has been of the highest quality.

As a medical society, this organization was first known as the American Sanatorium Association from 1905 to 1939. At this latter date, because of its broadened activities in the field of thoracic diseases, the name was changed to the American Trudeau Society. Even in 1939 there was strong opposition to giving this society a name that memorialized an individual rather than one that embodied the true nature of the work of its members. But the name Trudeau stuck for a variety of reasons. Today the changing picture of the control and distribution of tuberculosis, as well as the emergence of a host of nontuberculous diseases of the chest, has again made another change of name both urgent and needed. It is fair to say that even full-time tuberculosis sanatorium physicians are exploring every phase of thoracologic medicine and all of its allied specialties. The United States Public Health Service has clearly delineated the changing picture in the care of tuberculosis: from the sanatorium to the home, and from the specialist to the internist and the family doctor.

Thus, the structure and the function of the Trudeau Society has broadened, not only in the field of tuberculosis, but in the realm of nontuberculous disease of all kinds: fungal and saprophytic infections; industrial pulmonary disease; environmental pathogens; air pollution; cardiopulmonary physiology; the bacteriology of chronic bronchitis; cancer; and the very important degenerative diseases, of which obstructive emphysema poses most difficult problems. And still more far reaching is the pathology of the lungs as a manifestation of systemic bodily disease. For these reasons it appears that the time is ripe for a change of name from Trudeau to one that embodies these wider concepts of our function as a medical society.

No change of name, however, is logical without coincident change in the structure of our organization. Only those changes in structure and function are being considered which will make it a more democratic organization and which will give national representation from all of the state societies to the central governing councils. A larger council is pictured, composed of one representative from each state Trudeau Society plus fifteen members-at-large. The latter might be chosen by the nominating committee of the American Trudeau Society and approved by the whole Council. It is hoped that the representative members, elected by popular vote for their recognized accomplishments in thoracic medicine or surgery will be true representatives of their state societies at the Council meetings of the American Trudeau Society. In turn, they should be responsible

to their respective states for their contributions to the administration of policy. Such a Council should reflect the regional trends and activities of its members. It should give strength to the Trudeau Society with such new and wider representation. It should present greatly broadened opportunity for young and old from all parts of the country to be seen and heard concerning the administrative plans for the present and the future of the Society. The purpose of this new Council is to present the young doctor with a progressive medical society in thoracologic disease. With such a Council there should be tangible opportunity for growth in directions which will embody the practicing physician and surgeon as well as the full-time sanatorium and hospital doctor. A small Executive Committee will function on administrative matters between the meetings of the larger Council.

Whatever name is finally chosen, whether it remain Trudeau or become Thoracologic or Thoracic, the decision should be for the soundest and most promising future for the American Trudeau Society. This future we envision as tending more and more toward the needs of the practicing doctor in his greatly broadened responsibilities in thoracic disease, along with a name that reflects in his own mind and that of his fellow doctors and patients and friends the nature of the work in which he is engaged.

Above all, however, "United we stand . . ." in carrying on the great work of a great Society. Our members in past years have set the pattern of high ideals in the practice of thoracic medicine as well as leadership in education and integrity in research. So we, with a more appropriate name and a more representative Council, may grow with the ever-changing times and be true to the youth of our profession. Our Society is one that should continue to expand into new and modern fields of thoracic disease, and no imagination should be spared in planning and plotting our future course.

THEODORE L. BADGER
Chairman, Special Committee on Organizational Structure of the American Trudeau Society[1]

[1] Other members of Special Committee on Organizational Structure of the American Trudeau Society: William L. Cooke, Bruce E. Douglass, Edward A. Gaensler, William M. M. Kirby, and Roger S. Mitchell.

Notes

EFFECT OF EARLY ADMINISTRATION OF ISONIAZID ON THE IMMUNIZING ACTIVITY OF NORMAL BCG AND ISONIAZID-RESISTANT BCG IN GUINEA PIGS

Theoretical considerations developed elsewhere[1] have prompted the selection of an isoniazid-resistant strain of BCG and the study of its immunizing activity in comparison with a standard BCG strain. The study has been made with both vaccines, using two groups of vaccinated guinea pigs, one receiving isoniazid during the period between the vaccination and the challenge infection; the other receiving no isoniazid. In addition, a special control group of guinea pigs vaccinated with heat-killed BCG was used.

Preparation of isoniazid-resistant BCG: The isoniazid-resistant BCG strain has been developed by four successive transfers of a standard BCG strain on Löwenstein-Jensen medium containing 1 γ of isoniazid per ml. for the first transfer, 5 γ per ml. for the second transfer, 10 γ per ml. for the third and fourth transfers. The second transfer on Löwenstein-Jensen medium containing 10 γ of isoniazid per ml. yielded an abundant growth which was identical to that of a standard BCG culture. The culture was used for the vaccination of the animals. Plating of suitable dilutions demonstrated that *all* of the bacilli were resistant to at least 10 γ per ml. The isoniazid-resistant BCG strain used is catalase negative (Middlebrook[2] technique), whether taken from a tube of medium containing isoniazid or from a tube without isoniazid. The original BCG strain is strongly catalase positive.

Vaccination and administration of isoniazid: The vaccination study included 100 adult guinea pigs, weighing between 650 and 850 gm. One group of 40 guinea pigs received intramuscularly in the right thigh a saline suspension of 1 mg. (wet weight) of the twenty-four-day-old *isoniazid-resistant* BCG culture described above. On the same day a second group of 40 guinea pigs received a similar dose of the *standard* BCG strain from which the isoniazid-resistant BCG strain had been developed; the inoculum was taken from a twenty-two-day-old Löwenstein-Jensen subculture. A third group of 20 guinea pigs was injected intramuscularly with 1 mg. of heat-killed standard BCG in saline suspension.

For eighty-one days following the vaccination, 20 guinea pigs from the first group and 20 from the second group received daily a subcutaneous injection of 5 mg. of isoniazid. On the day after the treatment was discontinued, all of the animals of the three groups were tested intradermally with 10 units of purified tuberculin.

Challenge infection: Three days after the administration of isoniazid was discontinued, eighty-four days after vaccination, all of the 88 surviving guinea pigs were infected, by intramuscular injection in the left thigh, with 1 ml. of a dilution of a nine-day-old culture of the virulent strain H37Rv in Dubos liquid medium, adjusted by turbidimetry to a concentration of 1:500 mg. of bacilli per ml. The plating of appropriate dilutions showed that 52,000 viable bacilli had been inoculated per animal. On the same day, 30 control guinea pigs received the same virulent inoculation. None of the animals, vaccinated or control, received isoniazid during the evolution of the challenge infection.

All of the 83 surviving vaccinated guinea pigs and 18 controls were sacrificed ninety-nine to one hundred and twelve days after the challenge, the average day of sacrifice for each group being the one hundred and fifth day. Two guinea pigs vaccinated with killed BCG and 2 controls died spontaneously at the same time and were added to the sacrificed animals. Previously, only one animal, vaccinated with killed BCG and superinfected, died of tuberculosis.

[1] CANETTI, G.: Rev. de la tuberc., 1955, *19*, 1392; Am. Rev. Tubere., 1956, *74* (Supplement, p. 13).

[2] MIDDLEBROOK, G.: Am. Rev. Tubere., 1954, *69*, 471.

At the autopsy of each animal, the local satellite lymph nodes (left internal and external lymph nodes and lumbar lymph nodes) were weighed together. The degree of macroscopic involvement of the spleen, liver, and lung was evaluated by an index from 0 to 5 for each organ; the degree of involvement of the local lymph nodes, taken together, by an index from 0 to 3. The *total* macroscopic involvement of the animal was thus measured by an index of 0 to 18 (*total index*).

* * *

The results of the experiment are to be considered for the following six groups of animals:

Group *S.T.*: Guinea pigs vaccinated with standard BCG and treated with isoniazid (20 animals)

Group *S.NT.*: Guinea pigs vaccinated with standard BCG and not treated with isoniazid (16 animals)

Group *I.T.*: Guinea pigs vaccinated with isoniazid-resistant BCG and treated with isoniazid (16 animals)

Group *I.NT.*: Guinea pigs vaccinated with isoniazid-resistant BCG and not treated with isoniazid (17 animals)

Group *K.*: Guinea pigs vaccinated with killed BCG (16 animals)

Group *C.*: Control guinea pigs (20 animals)

Allergy produced by the vaccination: The intradermal tuberculin reactions elicited before the challenge, on the eighty-second day after vaccination, were read at twenty-four and forty-eight hours. The maximal diameter of the reaction at forty-eight hours was as follows (average of each group):

Group *S.T.*: 8.2 mm. Group *S.NT.*: 12.0 mm.
Group *I.T.*: 12.4 mm. Group *I.NT.*: 13.5 mm.
Group *K.*: 2.3 mm.

The percentage of reactions with a necrotic center of the total number of reactions for each group was as follows:

Group *S.T.*: 5% Group *S.NT.*: 25%
Group *I.T.*: 41% Group *I.NT.*: 53%
Group *K.*: 0%

Thus, the allergy was weak in the group vaccinated with the killed BCG. The allergy displayed by the group vaccinated with the standard BCG and not treated with isoniazid was strong. It was practically equivalent to the allergy displayed by both groups vaccinated with isoniazid-resistant BCG, whether treated or not. The allergy in the group vaccinated with standard BCG and treated with isoniazid was weaker than that of the three preceding groups. The difference is significant; however, it is not very large. The allergy in this group was far stronger than that of the group vaccinated with killed BCG.

Weight changes during the challenge infection: The changes in weight between the day of the challenge and the day of sacrifice averaged as follows:

Group *S.T.*: −5% Group *S.NT.*: 0%
Group *I.T.*: +5% Group *I.NT.*: −1%
Group *K.*: −11% Group *C.*: −11%

None of these changes is significant.

Tuberculous involvement induced by the challenge infection: The involvement of the *local lymph nodes* was maximal for the control groups (average weight 1.9 gm.) and for the group which received killed BCG (1.6 gm.); almost identical for group *S.T.* (1.5 gm.); somewhat less for group *S.NT* (1.3 gm.); and notably less in the two groups *I.NT.*

TABLE 1

ALLERGY AND IMMUNITY INDUCED IN GUINEA PIGS BY STANDARD BCG AND ISONIAZID-RESISTANT BCG IN THE PRESENCE AND IN THE ABSENCE OF AN EARLY ISONIAZID TREATMENT

Group*	Number of Animals	Allergy†		Immunity‡					
				Variation in Mean Weight			Macroscopic Involvement		
		Mean Diameter	Per Cent of Necrotic Reactions	Before§	After‖	Variation	Spleen¶	Total Index**	Visceral Index††
		mm				*per cent*			
S.T.	20	8.2	5	750	710	−5	2.3	6.4	4.6
S.NT.	16	12.0	25	760	760	0	1.8	5.5	3.7
I.T.	16	12.4	41	760	800	+5	1.1	2.1	1.5
I.NT.	17	13.5	53	820	810	−1	1.1	1.8	1.1
K.	16	2.3	0	760	670	−11	2.9	9.5	7.2
C.	20			760	680	−11	3.2	9.2	7.1

* For explanation of the letters *see* text.

† Animals tested intradermally with 10 units of purified tuberculin, 81 days after vaccination. Results at 48 hours.

‡ Challenge, 84 days after vaccination, by intramuscular injection of 52,000 viable bacilli of H37Rv. Sacrificed 99 to 112 days later.

§ Mean weight on the day of challenge.

‖ Mean weight on the day of sacrifice.

¶ Weight in grams.

** Macroscopic involvement estimated 0 to 5 for the spleen, 0 to 5 for the liver, 0 to 5 for the lungs, 0 to 3 for the local lymph nodes: 0 to 18 for the whole animal.

†† Same index, excluding the local lymph nodes.

(0.53 gm.) and *I.T.* (0.45 gm.). The majority of the animals of the last two groups showed no significant necrosis in the local lymph nodes.

The most important criteria for evaluating the tuberculous involvement induced by the challenge infection are *the weight of the spleen* and *the total index* (table 1). The weight of the spleen was highest for groups *C.* (3.2 gm.) and *K.* (2.9 gm.); less for group *S.T.* (2.3 gm.); still less for group *S.NT.* (1.8 gm.), and minimal for groups *I.NT.* (1.1 gm.) and *I.T.* (1.1 gm.). If these weights are related to the total weight of the animal at the moment of sacrifice, the trend of the results remains the same, but the difference between the groups is somewhat accentuated.

The total index (maximum = 18) was highest in groups *K.* (9.5) and *C.* (9.2); less in group *S.T.* (6.4); still less in group *S.NT.* (5.5); and very low in groups *I.T.* (2.1) and *I.NT.* (1.8). The trend of the results is the same as that displayed by the weight of the spleen.

A different coefficient can be considered, excluding local lymph nodes and based only on the degree of involvement of the spleen, liver, and lung, i.e., the degree of hematogenous generalization of the infection. This criterion is justified by the fact that, in guinea pigs, even bacilli of low virulence are able to produce large local lymph node involvements; only early and nonregressive visceral involvement demonstrates the severity of a tuberculous infection. The results obtained with this *visceral coefficient* (maximum = 15) are indicated in table 1. The trend of the results remains the same; but those shown by the two groups vaccinated with standard BCG approach more closely those of the

two groups vaccinated with isoniazid-resistant BCG than they do with the preceding criterion.

To summarize, the results of this vaccination experiment remain practically the same regardless of the criterion used to evaluate the immunity induced by the vaccination. There was no specific immunity in the group vaccinated witd killed BCG, the lesions being the same as in the control animals. Immunity was the strongest in the two groups vaccinated with isoniazid-resistant BCG, and there was no significant difference between the treated and untreated animals. In the two groups vaccinated with standard BCG, immunity was intermediary; it was stronger than in the control animals and those vaccinated with killed BCG but weaker than in animals vaccinated with isoniazid-resistant BCG. The slight difference between the treated and the untreated animals was not statistically significant.

* * *

Among the results reported here, those to be examined more closely deal with the difference in immunity induced by killed BCG and standard BCG opposed by an isoniazid treatment; the difference in immunity induced by standard BCG and isoniazid-resistant BCG; and, finally, the influence of treatment with isoniazid on the immunity induced by standard BCG.

In the first place, the immunity induced by standard (isoniazid-susceptible) BCG in animals *treated by isoniazid during the development of the vaccinal immunity* is much stronger than that induced by the same dose of heat-killed BCG. It is known from numerous experiments that even much larger amounts of killed BCG or killed virulent bacilli induce but a low level of immunity in guinea pigs. Since the adverse effect on the immunizing ability of bacilli achieved by killing the bacilli by heat (110°C.) is so much more pronounced than the adverse effect achieved by an isoniazid treatment, it is reasonable to assume that, whatever the number of bacillary units eventually involved in the immunizing process, heat killing produced a *denaturation* of the immunizing bacillary constituents and that no comparable denaturation occurs in bacilli antagonized by isoniazid. Differences in *speed* at which the constituents are released may also play a significant role.

In the second place, in these experiments, the immunizing capacity of isoniazid-resistant BCG appeared to be greater than that of standard BCG. It is known[3] that there are appreciable differences in immunizing capacity between different strains of BCG. But in these experiments, the isoniazid-resistant BCG was compared to the standard BCG strain from which it had originated; a difference existing *at the onset*, before the four passages on the isoniazid-containing media, can thus be excluded. Although the differences observed were statistically significant, it would be an overstatement to say that the difference in the immunizing capacity of the two strains is definitely proved, especially if one takes into account the uncertainties existing in challenge experiments when the infecting dose is not very high. It can merely be stated as yet that the isoniazid-resistant, catalase-negative BCG strain used here induced a satisfactory degree of immunity.

In the third place, the influence of the administration of isoniazid *from the moment of vaccination* on the allergy and immunity produced by standard BCG deserves special attention because of its bearing on two important problems, that of the general mechanism of immunization and that of a possible association of vaccination and chemoprophylaxis in the prevention of tuberculosis. In guinea pigs vaccinated with standard BCG and treated by isoniazid, *allergy is* weaker than in guinea pigs identically vac-

[3] Dubos, R. J., and Pierce, C. H.: Am. Rev. Tuberc., 1956, *74*, 699.

cinated and not treated. The difference, although statistically significant, is not very impressive: it is less pronounced than that noted by Choremis and his collaborators[4] in a similar experiment with children. As for *immunity*, the difference between the two groups of guinea pigs falls far below the level of statistical significance. A recent experiment of Dubos and Schaefer[5] demonstrates that, in mice infected by a *small* number of virulent bacilli, an immediate *intensive* treatment with isoniazid inhibits strongly the development of immunity; but there is no inhibition of immunity if the number of injected virulent bacilli is high.

The absence of a pronounced inhibitory influence of isoniazid on the immunizing activity of standard BCG, under the present experimental conditions, can be explained by several factors, all of which are still hypothetical. In the first place, since the antibacterial activity of isoniazid is closely linked with the phase of multiplication of the bacilli,[6] it may be supposed that the action displayed *in vivo* by isoniazid on an attenuated strain like BCG, which does not multiply very actively in the host, is less pronounced than the action displayed on a virulent strain, which does multiply actively in the host. There are as yet no precise studies available on the fate of BCG in guinea pigs treated by isoniazid. In men, Aronson and associates[7] observed that isoniazid treatment started forty-seven days after an intradermal BCG vaccination did not alter significantly the evolution of the local ulcer.

In the second place, the standard BCG inoculated contained a proportion of 10^{-4} mutants resistant to 0 γ1 of isoniazid per ml., 10^{-5} mutants resistant to 5 γ per ml. Plate counts of the inoculum (1 mg.) indicated the presence of 3×10^6 viable bacilli; each guinea pig inoculated with 1 mg. of standard BCG had thus received 300 bacilli resistant to 0 γ1 of isoniazid per ml. among which 30 bacilli were resistant to 5 γ per ml. Since the challenge infection was given only eighty-four days after the vaccination, it cannot be excluded that the few inoculated isoniazid-resistant BCG mutants played a role in building up the vaccinal immunity.

Lastly, setting aside the question of the survival of BCG *in vivo* under an isoniazid treatment, it may be supposed that the amount of immunizing constituents contained in 3×10^{-6} bacillary units is by itself sufficient to produce a satisfactory immunity, provided that no brutal denaturation by an action such as heating at 110°C. takes place.

The bearing of the reported results on the problem of a possible association of vaccination and chemoprophylaxis in the prevention of tuberculosis cannot be indicated as yet with certainty. The utility of an isoniazid-resistant BCG vaccine has been postulated[1] on the following theoretical grounds. Persons not yet infected by the tubercle bacilli and submitted to prophylactic administration of isoniazid would probably be left without any specific resistance at the moment of discontinuance of chemoprophylaxis. Vaccination should be performed on such persons, as they are supposed to live in a highly infective environment; and the vaccination should be realized during the administration of isoniazid, since protection afforded by BCG vaccination in men does not reach a satisfactory level before two to four months. Since standard BCG is susceptible to isoniazid, however, the possibility exists that the development of immunity is antagonized by the administration of isoniazid: hence the usefulness of vaccination by an isoniazid-resistant BCG.

The evidence resulting from this experiment does not indicate that the development

[4] Choremis, C., *et al.*: Am. Rev. Tuberc., 1956, *74* (Supplement, p. 197).
[5] Dubos, R. J., and Schaefer, W. B.: Am. Rev. Tuberc., 1956, *74*, 541.
[6] Schaefer, W. B.: Am. Rev. Tuberc., 1954, *69*, 125.
[7] Aronson, J. D., *et al.*: Am. Rev. Tuberc., 1956, *74*, 7.

of immunity due to a *high* dose of standard BCG would be significantly lowered by a simultaneous administration of isoniazid. It should be kept in mind, however, that the dosage of BCG used in human vaccination is far lower than that used in the present experiment: for instance, to a child weighing 15 kg. vaccinated intradermally by 1:20 mg. of BCG, the vaccinal dose given is only one-four hundredth of that given in this experiment. Further experimental evidence is necessary on the effect of isoniazid on the immunity induced by a guinea pig vaccination which would reproduce quantitatively the usual BCG vaccination in man.

In summary: A strain of isoniazid-resistant BCG has been prepared. The immunizing capacity of this strain in guinea pigs has been compared with that of standard BCG, in the presence and the absence of an immediate treatment by isoniazid, and with that of heat-killed BCG. Immunity was lowest in the group of animals vaccinated with heat-killed BCG. Immunity was highest in the group of animals vaccinated with isoniazid-resistant BCG; there was no difference between the treated and untreated animals. Immunity was intermediary in the group of animals vaccinated with standard BCG; in this group, the treatment lowered significantly the vaccinal allergy, but did not lower significantly the vaccinal immunity.

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COMPARATIVE STUDY OF THE VACCINATING PROPERTIES OF BCG AND ITS ISONIAZID-RESISTANT MUTANT IN GUINEA PIGS

Canetti[1, 2] suggested vaccination with an isoniazid-resistant strain of BCG and simultaneous treatment with isoniazid for a limited time as a method for the prevention of tuberculosis in children living in a tuberculous environment. Treatment with isoniazid would protect against tuberculous infection which might occur before immunity was established and yet would not interfere with its appearance since the strain used for the vaccination was resistant to the drug. The experiments described here deal with the question whether the isoniazid-resistant mutants used in this method possess a vaccinating power similar to that of the parent BCG strain (Phipps).

One group of 20 guinea pigs was vaccinated by subcutaneous injection of 0.5 ml. of a tenfold diluted ground and filtered suspension of a culture of BCG in Tween®-albumin medium. Another group of 20 guinea pigs was vaccinated with the same amount of a culture of the isoniazid-resistant, catalase-negative mutant of the same strain. The vaccinating doses contained 10^6 cultivatable bacterial cells. Six weeks later, the animals were tested with tuberculin 1:10. The average diameter of the area of redness was 14.4 mm. in the group vaccinated with the isoniazid-susceptible BCG and 10.6 mm. in the group vaccinated with the mutant strain. This difference was not statistically[3] significant ($p > 0.05$). However, 10 of the BCG-vaccinated animals and none of those vaccinated with the mutant strain showed a tuberculin reaction with central necrosis. Three days later the vaccinated animals and 20 control animals were challenged by exposure to pathogenic human tubercle bacilli (Pearson I) in the air-borne infection chamber devised by Middlebrook.[4] Four weeks later, 5 simultaneously infected animals of each group were sacrificed. The control animals showed numerous tubercles in their lungs, enlarged spleens, and tubercles on the liver, whereas the vaccinated animals showed fewer tubercles in their lungs and had spleens of normal size. In 2 animals vaccinated with the isoniazid-resistant strain, a few tubercles were seen on their spleens. Five control animals died after eighty-five, eighty-six, eighty-seven, ninety-three, and ninety-six days with the signs of generalized tuberculosis. One animal of each of the two vaccinated groups died after sixty-seven and seventy-eight days, and both presented extensive tuberculosis of the lungs and a few tubercles of the spleen. The experiment was terminated four months after challenge. At this time, the infection was generalized in all control animals and had also progressed in the vaccinated animals. No clear difference in the protective effect of the two BCG strains was apparent.

In a second experiment, two groups of 20 guinea pigs were vaccinated by two intradermal injections of 0.5 ml. of a filtered suspension of the two BCG strains containing 2×10^6 bacterial cells. The animals vaccinated with the isoniazid-resistant strain were given a solution containing 160 mg. of isoniazid per liter in their drinking water, beginning from the day of vaccination, for a period of two months. This dosage corresponded approximately to a daily intake of 8 to 10 mg. of isoniazid per kg. of body weight. Ten weeks after vaccination, all animals were found to react to tuberculin 1:50. The mean diameter of the tuberculin reactions was 15.25 mm. in the group vaccinated with the regular BCG and 13.5 mm. in the group of animals vaccinated with the mutant strain and treated with

[1] Canetti, G.: Am. Rev. Tuberc., 1956, *74* (Supplement, p. 20).
[2] Canetti, G.: Rev. de la tuberc., 1955, *19*, 1393.
[3] Ellis, M. G., and Heckstall-Smith, H. W.: Tubercle, 1955, *36*, 378.
[4] Middlebrook, G.: Proc. Soc. Exper. Biol. & Med., 1952, *80*, 105.

TABLE 1

NUMBERS OF COLONIES OF TUBERCLE BACILLI RECOVERED FROM GUINEA PIGS VACCINATED WITH ISONIAZID-SUSCEPTIBLE BCG, ISONIAZID-RESISTANT BCG, AND CONTROL ANIMALS AT FOUR WEEKS AFTER AIR-BORNE CHALLENGE INFECTION

Guinea Pigs	Vaccinated with BCG, Isoniazid Susceptible		Vaccinated with BCG, Isoniazid Resistant		Not Vaccinated	
	Lung	Spleen	Lung	Spleen	Lung	Spleen
1	2,200	30	6,000	130	60,000	4,000
2	3,300	200	400	230	34,000	5,000
3	90	0	—	0	100,000	4,000
4	800	30	600	1,500	24,000	3,000
5	240	10	700	40	56,000	3,000
Average....	1.3×10^3	5.4×10^1	1.9×10^3	3.8×10^2	5.5×10^4	3.8×10^3

isoniazid. This difference was not statistically significant. In the first group two reactions were necrotic; in the second, none. Three months after vaccination, the vaccinated and 20 control animals were challenged by air-borne infection with the pathogenic strain and four weeks later all animals were sacrificed. As in the previous experiment, all controls showed extensive tuberculosis of their lungs, enlarged spleens, and some tubercles on their livers, whereas the vaccinated animals, without noticeable difference between the two groups, showed somewhat fewer and smaller lung tubercles, spleens of nearly normal size, and no visible lesions on their livers. For a more objective evaluation of the involvement of the spleen this organ was weighed. The mean weight of the spleens of the control animals was 1.93 gm. It was 0.97 gm. in the animals vaccinated with the isoniazid-susceptible BCG, and 1.25 gm. in the group vaccinated with the mutant strain. The difference between the controls and the animals vaccinated with the isoniazid-susceptible BCG was highly significant ($p < 0.001$) and the difference between the controls and those vaccinated with the isoniazid-resistant strain was significant ($0.001 < p < 0.01$). The difference in the weight of the spleen of the two vaccinated groups was not significant ($p > 0.05$) (table 1).

In order to compare the degree of bacillary multiplication of the challenge strain in the vaccinated and the control animals, the right lower lobe of the lungs and one-half of the spleen of 5 animals from each group were homogenized in a Teflon® grinder in 5 ml. of 0.1 per cent bovine albumin; and 0.1 ml. of 10^{-1}, 10^{-2}, and 10^{-3} dilutions were plated in duplicate on oleic-acid–albumin-agar medium. The number of colonies recovered, corresponding to 0.1 ml. of the organ suspensions, is given in table 1. Statistical analysis of these data reveals that the difference in the counts from the lungs of the controls and the animals vaccinated with the isoniazid-susceptible BCG was highly significant ($0.001 < p < 0.01$). The difference in the counts from the lungs of the two vaccinated groups was not significant ($p > 0.05$). Similar results were obtained when the counts from the spleens were compared.

These results indicate that the immunizing activity of the isoniazid-resistant BCG strain is not strikingly different from that of the parent strain. This conclusion applies only to the vaccinating doses used, which were as large as those used for the vaccination of a human being or, on a comparative body weight basis, one hundred times larger. With smaller vaccinating doses the isoniazid-resistant BCG may provide significantly less protection than its parent strain. This possibility must be considered, especially in view of the finding that, on the average, the animals vaccinated with the isoniazid-resistant BCG

were less tuberculin hypersensitive than those vaccinated with the isoniazid-susceptible strain before challenge and, after challenge, developed larger spleens and higher levels of bacterial multiplication in this organ.

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THE INFLUENCE OF STREPTOVARICIN USED ALONE AND WITH ISONIAZID IN AN EXPERIMENTAL TUBERCULOUS INFECTION IN ANIMALS, AND SOME CLINICAL OBSERVATIONS[1]

Streptovaricin, a substance of microbial origin possessing antituberculous activity, has recently been discovered by workers in the Upjohn laboratories[2, 3] and the influence of this agent on tubercle bacilli *in vitro* and in experimental animals has been reported.[4]

The present report is concerned with observations on streptovaricin used singly or with either isoniazid or pyrazinamide in experimental tuberculous infections in mice. Certain clinical observations of the use of streptovaricin alone in tuberculous patients are likewise reported.

The microbial enumeration technique that was employed for the animal studies has been described previously.[5] In the previous studies it was demonstrated that otherwise drug-susceptible tubercle bacilli persisted in the tissues of experimentally infected animals after prolonged treatment with large doses of isoniazid, streptomycin, and para-aminosalicylic acid (PAS). This pattern was changed only when pyrazinamide was introduced into the test system.[6] When pyrazinamide was administered along with isoniazid, the tubercle bacilli vanished from the mouse tissues in so far as could be determined by microscopy, culture, or guinea pig subinoculation. After a treatment-free interval, tubercle bacilli could again be isolated from some of the animals. Thus the pyrazinamide-containing drug regimens had the unique property of producing a truly latent infection, i. e., one in which bacilli were present but were hidden beyond diagnostic reach.

In the present studies the microbial enumeration technique was used to evaluate the influence of streptovaricin administered alone and with isoniazid on populations of tubercle bacilli in lungs and spleens of mice during a twelve-week treatment period. Both the infection and the antimicrobial therapy were started on the same day. The results of this experiment may be seen in figures 1 and 2.

In the lungs of the untreated animals, the population of tubercle bacilli rose steadily during the first five weeks of the infection and remained high for the remainder of the eighty-four-day period of observation.

In the isoniazid-treated animals, the microbial populations fell promptly during the first three weeks. From that point on, the populations of tubercle bacilli in some of the lungs of animals treated with isoniazid alone were below the level of detectability.[7]

The population of tubercle bacilli in the lungs of animals treated with streptovaricin

[1] This study was supported in part by grants from the Navajo Tribal Council; the Committee on Medical Research of the American Trudeau Society, medical section of the National Tuberculosis Association, made possible by a special bequest from the estate of Sophie Goman; and by research grant E-635 from the Institute of Allergy and Infectious Diseases, National Institutes of Health, Public Health Service.

[2] SIMINOFF, P., *et al.*: Am. Rev. Tuberc., 1957, *75*, 576.

[3] WHITFIELD, G. B., *et al.*: Am. Rev. Tuberc., 1957, *75*, 584.

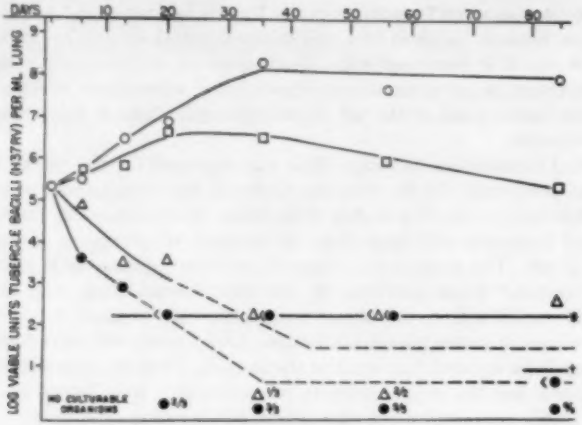
[4] RHULAND, L. E., *et al.*: Am. Rev. Tuberc., 1957, *75*, 588.

[5] MCCUNE, R. M., JR., AND TOMPSETT, R.: J. Exper. Med., 1956, *104*, 737.

[6] MCCUNE, R. M., JR., *et al.*: J. Exper. Med., 1956, *104*, 763.

[7] As is shown by the asterisk in figures 1 and 2, the conventionally used techniques permitted the detection of 75 to 90 culturable units of tubercle bacilli per organ. On day 84 of the experiment, however, as is shown by the dagger in the figures, a technique of increased sensitivity was substituted for the population enumeration studies in the animals treated with streptovaricin plus isoniazid. As few as 1 to 3 tubercle bacilli were theoretically detectable in each organ.

FIG. 1. Influence of streptovaricin and isoniazid used singly and together on populations of tubercle bacilli (H37Rv) in mouse lungs during 84 days of therapy. Infecting inoculum: 4.0×10^6 culturable units of tubercle bacilli: ○, control; □ streptovaricin; △ isoniazid; ● streptovaricin-isoniazid. Each symbol represents the average lung populations of 2 to 6 animals.



* The techniques used permitted detection of 70 to 90 culturable units of tubercle bacilli per lung.

† The techniques used on day 84 for streptovaricin-isoniazid animals permitted detection of 1 to 3 culturable units of tubercle bacilli per lung.

alone showed a definite increase during the first three weeks of treatment. This was followed by a gradual fall, but on the last day of the twelve-week treatment period, the microbial census in the lung was as high as it had been twenty-four hours after treatment was started. In contrast, when streptovaricin was administered with isoniazid, the bacterial populations were reduced markedly. After three weeks of therapy with both drugs, no organisms could be detected in the lungs of 2 of 3 animals. No tubercle bacilli could be cultured from any of the lungs in the treatment group during the remainder of the experiment. Despite the use of the more sensitive culturing techniques, on the eighty-fourth day of treatment no organisms could be detected in the lungs of any of the 6 animals examined.

In the spleens (figure 2) of untreated animals the population was similar to that presented in figure 1. Tubercle bacilli persisted throughout the entire twelve-week period of therapy with isoniazid alone. As in the lungs of animals treated with streptovaricin alone, the splenic populations of the same animals demonstrated a slight initial population increase. This was followed by a fall which continued gradually but steadily throughout the eighty-four-day treatment period. On the last day of the experiment tubercle bacilli could not be detected in the spleens of 2 of 5 animals.

The population pattern in the spleens of animals treated with streptovaricin and isoniazid together was of particular interest. The splenic census of tubercle bacilli was substantially reduced during the early weeks of therapy and continued to fall during the remainder of the twelve-week treatment period. In the third and fifth weeks of treatment

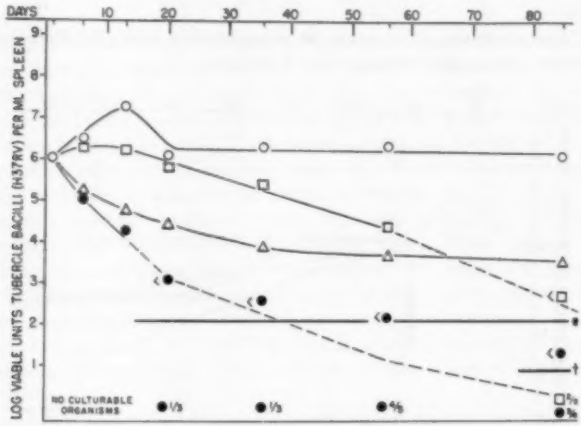
no tubercle bacilli could be cultured from the spleens of 1 of 3 animals examined. After eight weeks of therapy it was demonstrated that 4 of 5 animals had no culturable tubercle bacilli in the spleen. On the eighty-fourth day of treatment when techniques were used that permitted detection of 1 to 3 culturable units of tubercle bacilli, no organisms were present in the spleens of 3 of 6 animals.

When the start of isoniazid therapy used singly was postponed for a twenty-one-day period after the mice were infected with tubercle bacilli, the effectiveness of this agent on the populations of tubercle bacilli in the lung was considerably less than when treatment was started at the time of infection (figure 3). The use of streptovaricin alone resulted in approximately the same low degree of effectiveness on the pulmonic census of tubercle bacilli as was seen when treatment was initiated on the same day as the infection. Indeed, the microbial population was only slightly lower on the last day of treatment than on the day therapy was started. When streptovaricin and isoniazid were administered together, however, the effects were striking. The microbial populations in the lung fell steadily and after nine weeks of treatment tubercle bacilli could not be cultured from 3 of the 4 animals examined.

In the spleens (figure 4), after a slight initial increase in the bacterial populations, the effectiveness of streptovaricin when used alone in the twenty-one-day infection was equal to that seen with isoniazid alone. With both drugs the microbial census stabilized and the tubercle bacilli persisted throughout therapy. As was seen in the lungs of the same animals, the populations of tubercle bacilli in the spleens of animals treated with strepto-

FIG. 2. Influence of streptovaricin and isoniazid used singly and together on populations of tubercle bacilli (H37Rv) in spleens of the same animals whose lung populations are shown in figure 1.

○, control; □ streptovaricin; △ isoniazid; ● streptovaricin-isoniazid. Each symbol represents the averagesplenic population of 3 to 6 animals.

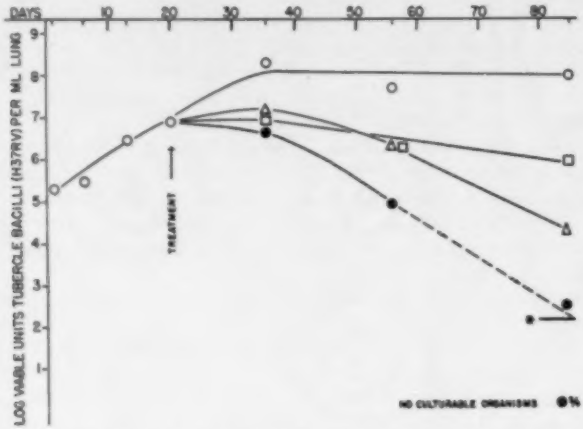


* The techniques used permitted detection of 70 to 90 culturable units of tubercle bacilli per spleen.

† The techniques used on day 84 for streptovaricin-isoniazid animals permitted detection of 1 to 3 culturable units of tubercle bacilli per spleen.

FIG. 3. Influence of streptovaricin and isoniazid used singly and together on populations of tubercle bacilli (H37Rv) in mouse lungs in the presence of lesions. Treatment was started 20 days after initiation of infection. Infecting inoculum 4.0×10^6 culturable units of tubercle bacilli:

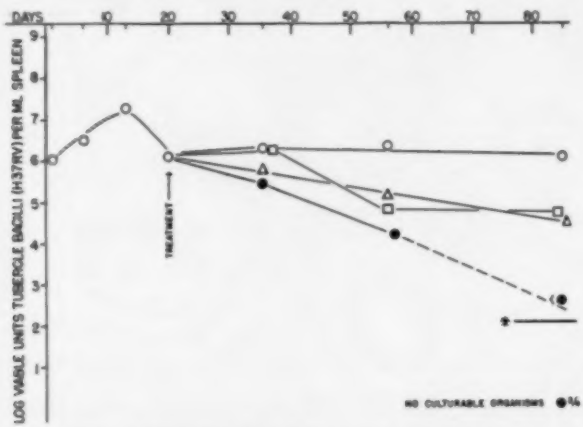
○ control; □ streptovaricin; △ isoniazid; ● streptovaricin-isoniazid. Each symbol represents the average lung population of 3 to 4 animals.



* The techniques used throughout this experiment permitted detection of 70 to 90 culturable units of tubercle bacilli per lung.

FIG. 4. Influence of streptovaricin and isoniazid used singly and together on populations of *M. tuberculosis* (H37Rv) in spleens of the same animals whose lung populations are shown in figure 3. Treatment was started 20 days after initiation of infection. Lesions were present.

○ control; □ streptovaricin; △ isoniazid; ● streptovaricin-isoniazid. Each symbol represents the average splenic population of 3 to 4 animals.



* The technique used throughout this experiment permitted detection of 70 to 90 culturable units of tubercle bacilli per spleen.

varicin and isoniazid together fell rapidly after initiation of treatment. No tubercle bacilli could be cultured from the spleens of 2 of 4 animals examined after nine weeks of treatment.

When treatment was initiated on the same day as infection, streptovaricin and pyrazinamide administered together were as effective as pyrazinamide–isoniazid[5] in reducing the lung and spleen populations of tubercle bacilli below levels of detection in all animals examined after twelve weeks of therapy.

* * *

The action of streptovaricin on the populations of tubercle bacilli in the tissues of the experimentally infected mice represented a pattern of antimicrobial influence which had not previously been observed in this experimental system. In contrast to isoniazid, for example, streptovaricin administered alone exhibited but a slight effect in reducing the populations of tubercle bacilli in the lungs throughout the whole period of treatment when the treatment was started on the day of infection. Moreover, in the spleens of the same animals the reduction in microbial census during the early weeks of the therapy was only slight. Yet after the third week of treatment the microbial census in the spleen began to fall and after eighty-four days of treatment it was lower in the streptovaricin-treated animals than in those treated with isoniazid. When streptovaricin was administered concomitantly with isoniazid, however, in situations when treatment was either initiated on the day of infection or delayed until lesions were established, the antituberculous effectiveness was considerably greater than that produced by either drug alone. In both experiments the tubercle bacilli were reduced below levels of detection in animals treated with both streptovaricin and isoniazid. The over-all results observed with streptovaricin and isoniazid administered together were definitely superior to any observed in this experimental system with other antituberculous drugs used singly or in combinations except for pyrazinamide-companion drug regimens.

* * *

Since August, 1956, a total of 17 patients have received streptovaricin on the New York Hospital and Navajo tuberculosis services. Six received streptovaricin principally to determine its tolerability and 11 received the drug for evaluation of its therapeutic effectiveness. A total daily dosage of 50 mg. per kg. of body weight was administered in two divided doses by the oral route. The 6 patients utilized in the studies of drug tolerability had active far advanced pulmonary tuberculosis despite prolonged previous treatment with the available antituberculous drugs. During the five months in which these patients received streptovaricin, the only untoward reaction observed was slight anorexia and nausea when the streptovaricin was given before meals. These mild gastrointestinal symptoms disappeared when the drug was again given with meals. Frequent laboratory tests of hepatic, renal, hematopoietic, and eighth-nerve function revealed no abnormalities during the observation period.

One of these 6 patients (*L. P.*) died following a severe pulmonary hemorrhage seven weeks after starting streptovaricin. There seemed to be no reason to attribute this death to the chemotherapy.

Of the 11 patients who have been treated with streptovaricin for therapeutic evaluation, 10 had moderately or far advanced pulmonary tuberculosis and one patient had acute miliary tuberculosis. Eight of the patients had never received any previous chemotherapy; 3 had received short courses of isoniazid or streptomycin in the past, but had not received either of these drugs in the several months prior to streptovaricin administration.

The clinical experience with streptovaricin treatment in these 11 patients is presented in table 1.

The 11 patients received the streptovaricin for various periods ranging from twenty-three to one hundred thirteen days. Seven of the 11 patients received the drug for more

TABLE 1

OBSERVATIONS ON STREPTOVARICIN USED SINGLY IN TUBERCULOUS PATIENTS

Patient	Type of Disease	Duration of Treatment in Days	Roentgenographic Improvement	Disappearance of Tubercle Bacilli from Sputum	Complications
1. A. M.	Pulmonary (FA)*	113	Slight	Yes	—
2. M. P.	Miliary	97†	Slight	No	Meningitis
3. L. A.	Far advanced	87†	None	No	—
4. N. S.	Moderately advanced	83	Slight	No	—
5. J. R.	Far advanced	75†	None	No	—
6. J. D.	Moderately advanced	72	Slight	No	—
7. M. W.	Far advanced	63†	None	No	Fever
8. O. R.	Far advanced	52†	Worse	No	Fever and chest pain
9. C. B.	Far advanced	49†	None	No	—
10. B. C.	Far advanced	43†	None	No	—
11. E. D.	Far advanced	23†	Worse	No	Fever

* Far advanced

† Streptovaricin discontinued

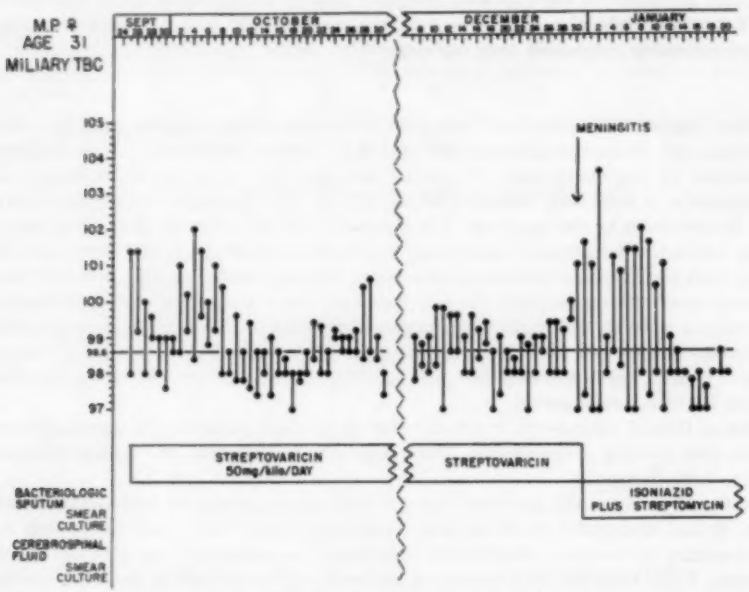


FIG. 5. Temperature record of *Patient M. P.*

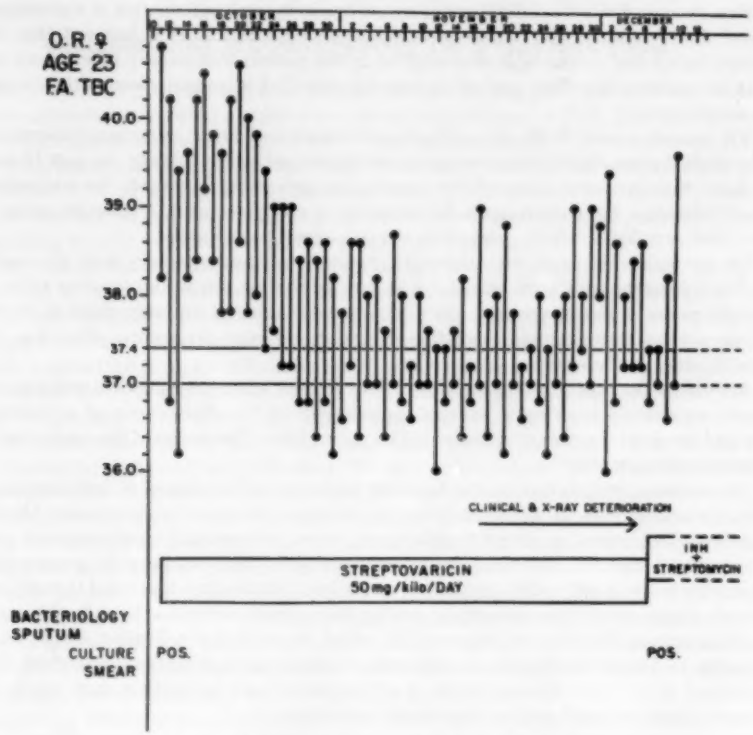


FIG. 6. Temperature record of *Patient O. R.*

than two months. Tubercle bacilli disappeared from the sputum of only one patient while receiving streptovaricin. Four patients were noted to have some roentgenographic improvement, but the remaining patients showed either no improvement or an actual worsening of the disease as determined by roentgenography.

Bacteriologic studies on the 11 patients revealed that all excreted tubercle bacilli after two months of streptovaricin therapy and in each case the cultures were susceptible to streptovaricin *in vitro*.

Two patients (*M. P.* and *O. R.*) were acutely ill on admission and their clinical course while on streptovaricin therapy is of special interest.

The first patient, *M. P.*, was a 31-year-old woman admitted with the characteristic syndrome of miliary tuberculosis. The temperature record of her clinical course is shown in figure 5. It may be seen that the patient slowly defervesced on streptovaricin therapy. Two febrile episodes during the first and last weeks of October were apparently the result of a nontuberculous infection of the urinary tract. The patient experienced a good initial clinical response and by the end of the third week of therapy appeared to be entering into a phase of complete clinical remission of the infection. The remission never became quite complete, however, and low-grade fever would appear intermittently. Moreover, the degree of roentgenographic clearing was only slight. On the ninety-sixth day of strepto-

varicin therapy the patient developed a severe headache, and examination of the cerebrospinal fluid confirmed the diagnosis of tuberculous meningitis. It is believed that the streptovaricin had a detectable clinical effect in this patient with miliary tuberculosis but that the extent of the effect was not comparable with that customarily seen with isoniazid or streptomycin.

The second patient, *O. R.*, was a 23-year-old woman with acute pulmonary tuberculosis who showed some slight clinical improvement on streptovaricin therapy. As may be seen in figure 6, defervescence occurred but required approximately four weeks for completion. Soon thereafter, the patient again developed fever and chest pain. A roentgenogram of the chest revealed a definite increase in the pulmonary tuberculosis.

On the basis of these clinical trials with streptovaricin and especially from the results in the 2 patients with acute disease, it is concluded that the antituberculous effect of streptovaricin in the dosage used was clinically detectable but was only slight in degree. It has not yet been determined whether this relatively poor therapeutic effect was due to difficulties in absorption.

Because of the high degree of antituberculous activity when streptovaricin and isoniazid were administered together in mice, a clinical study of the effectiveness of streptovaricin and isoniazid together was started in December, 1956. The results of this study will be reported subsequently.

In summary: Streptovaricin was found to have only a low degree of antituberculous effectiveness in mice as determined by the microbial enumeration technique. Despite this low effectiveness on streptovaricin alone, when streptovaricin and isoniazid were administered together, the total antituberculous effect was considerably greater than that achieved with either drug alone. From the clinical studies it is concluded that streptovaricin administered alone in a 50 mg. per kg. daily dosage exerted a clinically detectable antituberculous effect but the degree of the effect was only slight. Further studies are in progress to determine whether the substantial enhancement of total antimicrobial effect produced by the dual administration of streptovaricin and isoniazid in mice can be observed in patients with active tuberculous infections.

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CALCIUM BENZOYL PAS: REPORT OF A CLINICAL TRIAL[1]

The power of PAS to enhance the chemotherapeutic value of more powerful antituberculosis agents has stimulated a search for a better tolerated form of PAS. Recently, claims have been made that calcium benzoyl PAS[2, 3] satisfies this need.[4-7] Calcium benzoyl PAS is a white, barely bitter compound, only slightly soluble in water, which contains about 55 per cent of PAS by weight. When administered orally, the calcium is supposedly liberated in the stomach and the remainder is slowly absorbed from the small intestine.[8] The metabolism is to be considered in another paper from this hospital.

This communication deals with an attempt to learn how well patients tolerate this new compound as compared with sodium PAS. A preliminary trial[9] of calcium benzoyl PAS for four months failed to reveal any obvious toxicity. The following plan of study was then adopted. Patients with active pulmonary tuberculosis newly admitted to the service who were to receive PAS as a part of their chemotherapy were randomized by hospital number between sodium PAS and calcium benzoyl PAS. Both drugs were administered in powdered form, in similar containers, and in the same dose, i.e., 4 gm. three times daily. The powder, mixed with water at the bedside immediately prior to ingestion, was to be taken mid-way during each mealtime. Unfortunately, because the sodium PAS formed an almost colorless solution while the calcium benzoyl PAS became dispersed as a thin whitish mixture, the difference could not be concealed from the patients. Whenever it became necessary to discontinue either sodium PAS or calcium benzoyl PAS because of intolerance, an attempt was made to substitute the other drug in its place.

It soon became obvious that there were less complaints about the taste of calcium benzoyl PAS than of sodium PAS. Some patients who had to be switched to calcium benzoyl PAS after a trial with sodium PAS spontaneously commented on the more agreeable flavor of the former. The mere ingestion of medication was thus much less of a problem with calcium benzoyl PAS than with sodium PAS in this study.

No striking difference could be noted, however, in the number of adverse reactions which appeared with the two drugs. Most side-effects occurred within the first four months of treatment. A total of 96 randomized patients, treated for periods between four and eighteen months, are available for analysis (table 1). Of the 45 patients who received the sodium PAS, some intolerance was noted in 42 per cent, and it was necessary to stop therapy in 22 per cent. Of the 51 patients who received the calcium benzoyl PAS, intolerance was noted in 45 per cent, and it was necessary to stop therapy in 18 per cent. Of 14 patients

[1] Preliminary reports on the progress of this study were presented at the Fourteenth and Fifteenth Conferences on the Chemotherapy of Tuberculosis, Veterans Administration, Army, and Navy, held at Atlanta, Georgia, February 7-10, 1955, and St. Louis, Missouri, February 6-9, 1956, respectively.

[2] The calcium benzoyl PAS (Benzacyl) was kindly supplied for the purpose of this study by the Smith-Dorsey Division of the Wander Company, Chicago, Illinois.

[3] Hirt, R., and Hurni, H.: Helvet. chim. acta, 1949, *32*, 378.

[4] Steinlin, H.: Rapport à l'occasion de la 13ème conference de l'Union Internat. contre la Tuberc., Madrid, 1954, *26*, 9.

[5] Debray, J. R.: Presse méd., 1950, *58*, 647.

[6] Gow, J. G.: Brit. J. Urol., 1953, *25*, 316.

[7] Ross, J. C., *et al.*: Lancet, 1955, *1*, 116.

[8] Schonholzer, G., *et al.*: Schweiz. med. Wchnschr., 1955, *10*, 222.

[9] Phillips, S.: Tr. Fourteenth Conference on the Chemotherapy of Tuberculosis, Veterans Administration, Army, and Navy, 1955, p. 304.

TABLE 1

RANDOMIZED STUDY AFTER FOUR TO EIGHTEEN MONTHS' THERAPY

Drug	Number of Patients	Side-Effects		Drug Stopped
		Number of Patients	Number of Episodes	
Calcium benzoyl PAS	51	23 (45%)	36	9 (18%)
Sodium PAS	45	19 (42%)	38	10 (22%)

TABLE 2

SIDE-EFFECTS IN 91 PATIENTS ON CALCIUM BENZOYL PAS

	Number of patients
1. Abdominal discomfort	32
a. Varying degrees of pain	18
b. Nausea	18
c. Anorexia	5
d. Vomiting	3
2. Diarrhea	6
3. Pruritis ani	4
4. Dysuria	3
5. Skin irritation	2

unable to tolerate sodium PAS who were then given calcium benzoyl PAS, 9 could tolerate the calcium benzoyl PAS sufficiently to permit further therapy. Of 6 patients unable to tolerate calcium benzoyl PAS who were then given sodium PAS, 2 were able to tolerate the sodium PAS sufficiently well to permit further therapy.

Besides the randomized patients discussed above, there were a number of patients who were excluded from the study because of a history of past intolerance to PAS or sodium PAS and some who entered the hospital after the randomization process had been stopped. They made up a group of 29 patients who were treated for periods between four and twelve months with calcium benzoyl PAS, and who were studied mainly for information concerning toxicity. Side-effects were more common in these patients, as was to be expected, and were observed in 59 per cent. The side-effects were similar in character to those seen in the randomized group. It was necessary to stop treatment because of intolerance in 34 per cent of these patients.

The total experience with adverse symptoms from calcium benzoyl PAS is listed in table 2. The majority of adverse symptoms consisted of varying degrees of anorexia, flatulence, nausea, or abdominal cramping pain. Diarrhea was occasionally encountered, as was pruritis ani. A few patients complained of burning on micturition or of urinary frequency. Only rarely did a patient react to the medication with a skin eruption. There were no complaints of general malaise or of aching of the joints. Fever was noted in 5 patients, but a careful analysis of these cases indicates that the calcium benzoyl PAS probably was not the responsible agent for this symptom.

Of unusual interest were 3 patients who developed a constitutional sensitivity type of reaction when on sodium PAS which was then demonstrated to be due to this medication. These patients were found able to tolerate calcium benzoyl PAS shortly afterwards. It is

true that the latter was tried very cautiously, beginning with small "pinches" of drug which were gradually increased, but a similar trial with sodium PAS had evoked a return of symptoms in each case previously. The explanation for this unexpected finding is not known, but is mentioned for whatever worth it may have.

The laboratory tests employed in this study included a complete blood count, hematocrit, sedimentation rate, urinalysis, and thymol turbidity test of liver function on each patient at least once each month. A blood urea nitrogen determination was made at least every two months. The results of these tests need not be given in detail, for they all remained within limits of normal. There was thus no evidence in the patients of any toxic effect on the liver, kidney, or bone marrow.

The writers did not have the temerity to try calcium benzoyl PAS by itself as a therapeutic agent in the patients with active pulmonary tuberculosis. Such a procedure did not seem justifiable, so it is not possible to analyze the effect of calcium benzoyl PAS alone on the roentgenographic appearance of the lungs or on the bacillary content of the sputum. However, the ability of calcium benzoyl PAS to delay bacterial resistance to the streptomycin or isoniazid given concomitantly did seem impressive. Among 54 patients who received calcium benzoyl PAS for periods between four and eighteen months, there was not a single instance of the development of bacillary resistance to either streptomycin, isoniazid, or PAS by repeated *in vitro* examination.

In summary: This paper concerns an attempt to learn how well patients with active pulmonary tuberculosis tolerate calcium benzoyl PAS during long-term therapy as compared with sodium PAS. Most of the patients found calcium benzoyl PAS to be definitely more palatable than sodium PAS. However, as a group they experienced side-effects similar to those experienced with sodium PAS, and about as frequently. Some patients who could not tolerate sodium PAS were able to take calcium benzoyl PAS. The reverse also was true. There were 3 patients who had shown hypersensitivity manifestations with sodium PAS who were found able to continue with calcium benzoyl PAS. It was also found that this latter drug delayed bacterial resistance satisfactorily when given concurrently with streptomycin or isoniazid. It thus would appear that calcium benzoyl PAS is a safe, effective form of PAS, and that it should prove useful for some patients who cannot tolerate sodium PAS or who want a more palatable preparation.

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RELATION OF PYROGALLOL-PEROXIDATIVE ACTIVITY TO ISONIAZID RESISTANCE IN MYCOBACTERIUM TUBERCULOSIS[1]

Recent evidence indicates that the action of isoniazid on *Mycobacterium tuberculosis* may be related to iron-porphyrin enzymes which include the hydroperoxidases (catalase and pyrogallol-peroxidase). Isoniazid-resistant tubercle bacilli were found to be deficient in catalase activity[2] and, under certain conditions, catalase and hemin functioned as necessary factors for the growth of resistant organisms.[3, 4] The action of isoniazid on susceptible organisms was antagonized by hemin,[5] but this antagonism was later shown to result from nonbiologic inactivation of isoniazid.[6]

In view of the deficiency in catalase activity in isoniazid-resistant tubercle bacilli, concomitant reduction or elimination of peroxidative activity might be expected to occur in resistant organisms. Hence, determinations were made of the pyrogallol-peroxidative activity of normal tubercle bacilli and resistant organisms isolated from patients, as well as resistant, artificially induced strains of tubercle bacilli selected *in vitro*. The catalase activity of each organism was also measured.

The pyrogallol-peroxidative activity of *M. tuberculosis* was measured through an adaptation of the "purpurogallin method."[7]

In the adapted method, the components of the reaction mixture were: 2 ml. of 0.1 M phosphate buffer, pH 6.8; 1.5 ml. of 0.3 per cent hydrogen peroxide; 3.75 ml. of 0.1 per cent pyrogallol. Each organism was added to the reaction mixture in concentrations of approximately 10, 25, and 50 mg. (in terms of amount of culture removed from Löwenstein slant). In this procedure, the organisms were transferred to tared, screw-cap test tubes and weighed. Following addition of the buffer, the tubercle bacilli were emulsified thoroughly, then placed in an ice bath. Hydrogen peroxide was added followed by pyrogallol. The tubes were incubated at 37°C. for ten minutes, then returned to the ice bath, after which 0.15 ml. of 50 per cent sulfuric acid and 0.10 ml. of 20 per cent ascorbic acid were added to each tube. After centrifugation at 2,500 r.p.m. for fifteen minutes, 5 ml. of each supernatant were transferred to matched test tubes and the color was measured photometrically. From the readings on the colorimeter, the amount of purpurogallin formed in each tube was determined by means of a chart which had been prepared by measuring the color intensity of solutions containing increasing concentrations of purpurogallin. The intensity of color was directly proportional to the concentration of purpurogallin in the range of 5 to 100 γ per ml. The peroxidative values (purpurogallin, γ per ml.) for approximately 10, 25, and 50 mg. of culture of each organism were plotted (figure 1), and the value for 25 mg. was recorded as the pyrogallol-peroxidative activity of the organism. The pyrogallol-peroxidative activity was proportional to the weight of culture when the weight was not excessive.

For the determination of the catalase activity of *M. tuberculosis*, a modification of Peizer and Widelock's method[8] was used.

[1] A portion of this paper was presented at the meeting of the Society of American Bacteriologists, held in Houston, Texas, 1956.

[2] Middlebrook, G.: Am. Rev. Tuberc., 1954, *69*, 471.

[3] Cohen, M. L., *et al.*: Am. Rev. Tuberc., 1954, *70*, 641.

[4] Fisher, M. W.: Am. Rev. Tuberc., 1954, *69*, 797.

[5] Fisher, M. W.: Am. Rev. Tuberc., 1954, *69*, 469.

[6] Knox, R., and Woodroffe, R. C.: Brit. J. Exper. Path., 1955, *36*, 425.

[7] Theorell, H.: The Enzymes, edited by Sumner, J. B., and Myrback, K., Academic Press, Inc., New York, 1951, Vol. II, Part I, p. 397.

[8] Peizer, L. R., and Widelock, D.: Am. Rev. Tuberc., 1955, *71*, 305.

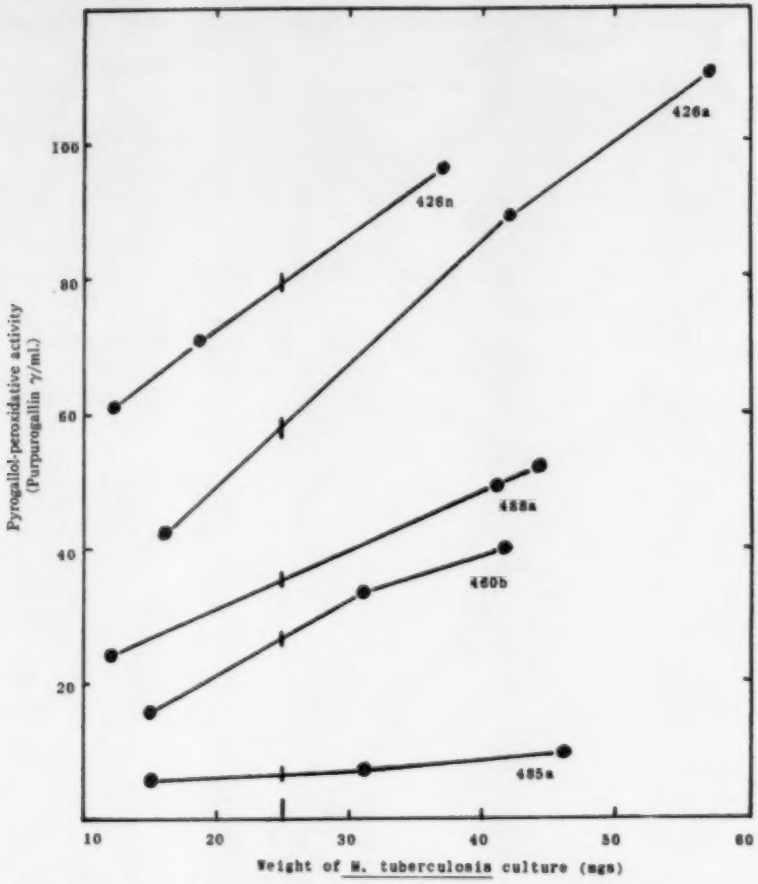

FIG. 1. Effect of varied amounts of normal and isoniazid-resistant cultures of *M. tuberculosis* on rate of pyrogallol-peroxidative reaction. Isoniazid susceptibility of each organism was as follows: 426*n*, 0.03 γ; 426*a*, 0.5 γ; 488*a*, 5 γ; 460*b*, 20 γ; 485*a*, 50 γ per ml.

The method was modified by increasing proportionally the amount of each reagent (phosphate buffer, hydrogen peroxide, citric acid, and ammonium molybdate) to yield a reaction volume of 7.5 ml., to which 50 mg. of each culture of tubercle bacilli were added. The concentration of hydrogen peroxide used in the test was decreased by one-half. The color which developed was measured photometrically, and the results were recorded in terms of amount of the hydrogen peroxide which was degraded.

In securing strains of *M. tuberculosis* with resistance to various amounts of isoniazid, the gradient plate method of Szybalski[9] was used.

The diffusion gradient plates were prepared by incorporating isoniazid into Herrold's medium which was modified by the addition of 0.5 per cent Trypticase® broth (BBL) and

[9] SZYBALSKI, W., AND BRYSON, V.: Am. Rev. Tubere., 1954, *69*, 267.

2 per cent human blood bank plasma. Ten strains of tubercle bacilli (series *n*), previously isolated from patients, were inoculated on diffusion gradient plates in which the lower wedge of agar contained 1 γ of isoniazid per ml. After incubation (for six weeks), organisms growing in the area (on each plate) which contained the highest concentration of isoniazid (series *a*) were suspended in Trypticase broth (BBL) and again inoculated on diffusion gradient plates containing 5 γ of isoniazid per ml. The isoniazid-resistant organisms from each plate were selected (series *b*) after incubation for six weeks.

Nine isoniazid-susceptible (series *n*) and 41 isoniazid-resistant strains of *M. tuberculosis*, 20 of which were selected *in vitro* (series *a* and *b*) and 21 of which were isolated from isoniazid-treated patients, were examined with respect to pyrogallol-peroxidative and catalase activity, as well as isoniazid resistance. Organisms used in determining the pyrogallol-peroxidative and catalase activity were cultured on Löwenstein medium for

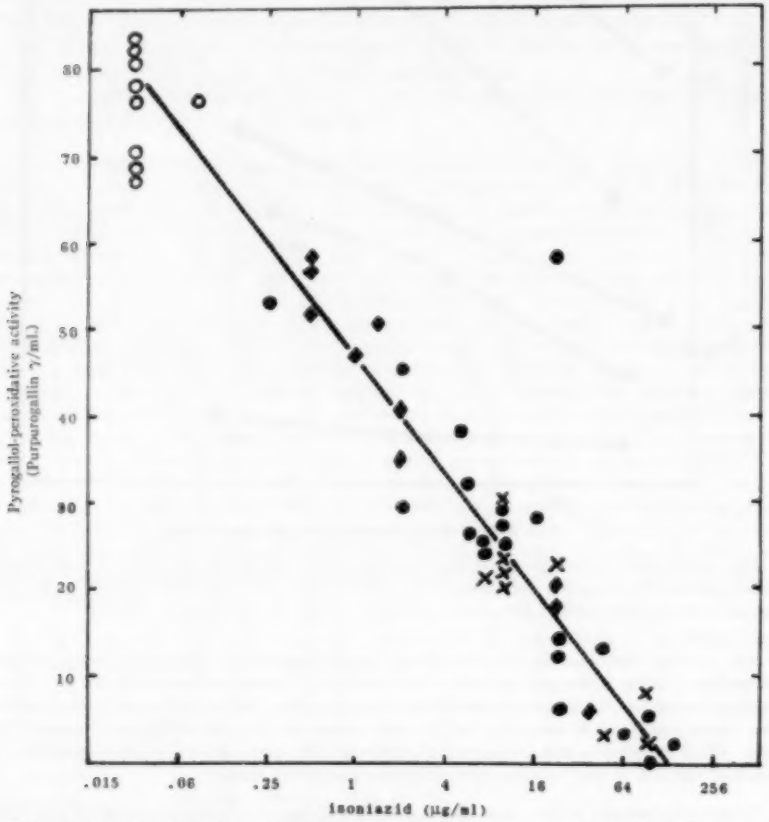

FIG. 2. Relationship of pyrogallol-peroxidative activity to isoniazid resistance in *M. tuberculosis*. ○ = normal organisms (series *n*); ◆ = resistant organisms selected *in vitro* (series *a*); × = resistant organisms selected *in vitro* (series *b*); ● = resistant organisms isolated from patients.

TABLE 1

CATALASE ACTIVITY OF NORMAL AND ISONIAZID-RESISTANT STRAINS OF MYCOBACTERIUM TUBERCULOSIS

Resistance of Various Strains of *M. Tuberculosis* (Isoniazid, γ/ml.)										
0.03	0.05	0.50	0.10	2.5	5.0	7.5	10.0	25.0	50.0	100.0
(Catalase Activity) (H_2O_2, γ/ml.)										
270	275	230	170	40	20	130	120	30	40	0
275	230	120		130	100	130	25	0	0	0
250		110			90	50	40	60	0	20
240					120	40	190	10	0	40
275						40	90	30		0
290							110	120		20
280										
220										
245										

three weeks. Resistance to isoniazid in terms of 50 per cent growth was recorded after the tubercle bacilli had been cultured for six weeks on Löwenstein medium containing varied amounts of the inhibitor. The relationship between pyrogallol-peroxidative activity and isoniazid resistance for each of the above organisms is recorded in figure 2. Resistance of the tubercle bacilli to isoniazid was a logarithmic function of the pyrogallol-peroxidative activity, with the exception of one organism.[10] There were no differences between the response of resistant organisms selected *in vitro* and the majority of resistant organisms isolated from patients. The relationship between catalase activity and isoniazid resistance is recorded in table 1. While catalase activity was, in each instance, indirectly related to resistance to isoniazid, there was considerable variation in the correlation of these values among individual organisms.

As the tubercle bacilli became resistant to isoniazid, their pyrogallol-peroxidative activity decreased until resistance to 100 γ of isoniazid per ml. was attained. At this level of isoniazid resistance, pyrogallol-peroxidative activity was absent in most instances, and the organisms displayed little or no catalase activity. The organisms which did not conform to this relationship exhibited a relatively high degree of pyrogallol-peroxidative and catalase activity despite resistance to isoniazid. Infrequent occurrence of a similar relationship between catalase activity and isoniazid resistance has been observed by other investigators.[3] Such deviations would appear to preclude the use of determinations of pyrogallol-peroxidative or catalase activity as an absolute means of predicting resistance of *M. tuberculosis* to isoniazid.

In all of the organisms investigated in this study, there was general agreement between pyrogallol-peroxidative and catalase activity in relation to isoniazid resistance. This finding may indicate that determination of pyrogallol-peroxidative and catalase activity measure two different functions of catalase in *M. tuberculosis* because this enzyme is now regarded as a special type of peroxidase which has the additional capacity to decompose peroxide in the absence of a second substrate.[7]

In summary: A method has been described for measuring the pyrogallol-peroxidative activity of *M. tuberculosis*. In a majority of strains it was found that resistance to iso-

[10] The catalase activity of this organism was 115.

niazid was a logarithmic function of the pyrogallol-peroxidative activity. There was general agreement between pyrogallol-peroxidative and catalase activity in relation to isoniazid resistance.

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[11] The writers express their appreciation to Mr. James Allen of Veterans Administration Hospital, Excelsior Springs, Missouri; and to Mr. James Burroughs, Veterans Administration Hospital, Augusta, Georgia, for supplying isoniazid-resistant cultures of *M. uberculosis*.

STUDIES ON NONTUBERCULOUS ACID-FAST BACILLI RECOVERED FROM HUMAN SOURCES[1]

II. Penicillin Susceptibility

Several investigators have shown that growth of typical strains of *Mycobacterium tuberculosis* is not inhibited by moderate concentrations of penicillin. Abbott, in 1951,[2] and Fallin and associates, in 1952,[3] added penicillin to sputum concentrates to reduce contamination and increase the number of cultures obtained that were positive for *M. tuberculosis*. The latter writers found, however, that penicillin in the amount of 400 units per ml. reduced the number of tubercle bacilli that could be recovered, but stated that this reduction was probably not important in specimens containing large numbers of bacilli.

Numerous other workers, Tarshis and his co-workers, 1951 and 1953[4, 5]; Whalen and Mallman, 1955[6]; Zuckerman and Rantz, 1951[7]; and Smith and his associates in 1949[8], have recommended the routine addition of penicillin to culture medium for the primary isolation of *M. tuberculosis*.

Kirby and Dubos in 1947[9] showed that pure cultures of tubercle bacilli are not inhibited by concentrations of penicillin that inhibit many other susceptible microorganisms. They reported the effect of the constituents of the medium upon the inhibitory effect of penicillin and showed that the presence of Tween® markedly increased the susceptibility of the tubercle bacillus to penicillin but, in the absence of Tween, 100 units of penicillin per ml. caused no inhibition of growth. Engbaek in 1954[10] suggested that, since virulent tubercle bacilli are able to grow in moderate concentrations (100 units of penicillin per ml.), this might be a means of differentiating between the typical virulent acid-fast bacilli and atypical or saprophytic mycobacteria. In an analysis of methods of differentiating between tubercle bacilli and acid-fast saprophytes, he found that the use of media containing 100 units of penicillin per ml. gave the most valuable information. When inhibition of growth occurred, the organism was probably a saprophyte. Tarshis and his colleagues in 1955[11] reported upon a group of chromogenic acid-fast bacilli from human sources. These acid-fast bacilli grew readily upon medium containing penicillin.

The present report deals with the *in vitro* penicillin susceptibility of these 33 strains of acid-fast bacilli. Investigations concerning the penicillin susceptibility of these organisms were undertaken with the following objective in view. Determination of drug-resistance and drug-susceptibility patterns might be an aid in the classification of these organisms and in the determination of their relationship to disease in human beings and animals.

[1] This investigation was supported by a research grant (NONR-1063 (00)) from the Office of Naval Research.

[2] Abbott, J. N.: Am. J. Pub. Health, 1951, *41*, 287.

[3] Fallin, J. R., *et al.*: Am. J. Clin. Path., 1952, *22*, 703.

[4] Tarshis, M. S., and Frisch, A. W.: Am. Rev. Tuberc., 1952, *65*, 278; 289; 302.

[5] Tarshis, M. S., *et al.*: J. Bact., 1953, *66*, 448.

[6] Whalen, J. W., and Mallman, W. L.: Am. Rev. Tuberc., 1955, *71*, 382.

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TABLE 1

GROWTH OF MYCOBACTERIA ON OLEIC-ACID–ALBUMIN AGAR WITH AND WITHOUT PENICILLIN

	100 Units of Penicillin	No Penicillin
11 atypical mycobacteria	++ to ++++	++++
22 atypical mycobacteria	0	++++
M. butyricum	0	++++
M. tuberculosis H37Rv	++++	++++
M. tuberculosis H37Ra	++++	++++
M. phlei	++++	++++
BCG-Tice	++++	++++

0 No growth
++++ Maximal growth

During a chest roentgenographic survey in the state of Colorado in 1949, sputum and gastric washings from 1,474 persons were examined bacteriologically. One hundred and seventy-four positive cultures containing acid-fast bacilli were obtained from sputum and gastric washings. Of these, 141 were cultures of typical *M. tuberculosis* virulent for guinea pigs and 33 were regarded as atypical. These 33 atypical mycobacteria were acid fast, grew well on Petragnani or Löwenstein-Jensen medium, and did not produce fatal or progressive disease in guinea pigs. Some of them were chromogenic.

The cultures of atypical mycobacteria were maintained on Löwenstein-Jensen medium and were transferred to Dubos Tween-albumin liquid medium before testing. Oleic-acid-albumin agar was used as the medium upon which penicillin susceptibility was determined. This was prepared as slants according to Dubos and Middlebrook in 1947.[12] After autoclaving, crystalline potassium penicillin G was added aseptically so that the final concentration was 100 units per ml. of medium. The control medium was prepared in the same manner without the addition of penicillin. Duplicate tubes of the penicillin-containing medium and also the penicillin-free medium were inoculated with the atypical strain and also with several known strains of mycobacteria. Inocula consisted of 1 loopful (2 mm.) of a fully grown culture in Dubos Tween-albumin medium. The cultures were incubated at 37°C. and examined daily. Final readings were made after twenty-eight days of incubation.

* * *

In table 1 it may be seen that, of the 33 strains of atypical mycobacteria studied, the growth of 22 strains was inhibited by 100 units of penicillin per ml. and the growth of 11 strains was not. All of the 11 strains whose growth was not inhibited by penicillin showed smooth deeply pigmented colonies on Löwenstein-Jensen medium and were regarded as typical "chromogens." Twenty of the 22 strains that were inhibited by penicillin did not present deeply pigmented colonies, although some were relatively smooth, while others were rough.

Timpe and Runyon in 1954[13] studied a large number of atypical acid-fast microorganisms and have divided them into three groups on the basis of colony form and color. *Group I* was composed of microorganisms having colonies that were grossly smooth, and buff or yellow in color. *Group II* was yellow-orange and smooth, while *Group III* was rough and buff to yellow. All groups were avirulent for guinea pigs, but *Groups I* and *III* were more virulent for mice than *Group II*.

The strains of atypical acid-fast bacilli in this study were grouped according to the classification suggested by Timpe and Runyon. It was found that the majority of strains

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[13] TIMPE, A., AND RUNYON, E. H.: J. Lab. & Clin. Med., 1954, *44*, 202.

TABLE 2

GROWTH INHIBITION OF ATYPICAL MYCOBACTERIA BY PENICILLIN

	Number of Strains Inhibited by Penicillin	Number of Strains Not Inhibited by Penicillin
Group I	15	0
Group II	2	11
Group III	5	0

belonging to *Group II* (yellow-orange, smooth, and less virulent for mice) were not inhibited by penicillin. On the other hand, those strains comprising *Groups I* and *III* (more virulent for mice) were inhibited by penicillin. The grouping and penicillin inhibition of these strains are shown in table 2.

It is evident from the table that the majority of strains that were capable of growth in medium containing 100 units of penicillin per ml. belong to *Group II* of Timpe and Runyon. These comprised a rather homogeneous group which formed yellow colonies that became deeper yellow or orange upon aging and were predominantly smooth. On the other hand, the majority of organisms that were inhibited by penicillin fell into *Groups I* and *III*. The colonies of these organisms were buff to yellow, those in *Group I* being predominantly smooth and those in *Group III*, rough. Several of the cultures falling into *Group III* could not be distinguished grossly from typical virulent tubercle bacilli.

Of the ten strains of chromogenic acid-fast bacilli reported by Tarshis and his coworkers,[11] all were able to grow in medium containing 100 units of penicillin per ml. Engbaek[10] found that only 19 of 47 strains of saprophytes were able to grow in penicillin medium, but this writer made no statement as to the colony color of his strains. From the results obtained by these investigators and by the above experiments, it is apparent that two general groups of acid-fast bacilli are capable of growth in medium containing moderate concentrations of penicillin: the typical virulent tubercle bacilli and the highly pigmented acid-fast saprophytes. These latter organisms are relatively avirulent for the mouse, according to Timpe and Runyon. Between these two extremes lies another group of organisms which are often grossly and microscopically indistinguishable from *M. tuberculosis*, but which are unable to grow in penicillin medium and which seem to be more virulent for mice, but not for guinea pigs.

In summary: In the foregoing experiments it has been shown that the majority of smooth chromogenic acid-fast bacilli, avirulent for guinea pigs, are able to grow in medium containing 100 units of penicillin per ml. Typical virulent tubercle bacilli are also able to grow in this medium. However, another group of acid-fast bacilli, many of which are morphologically indistinguishable from the tubercle bacilli, is unable to grow in penicillin medium. These organisms form an intermediate group since they are not chromogenic and might easily be confused with virulent tubercle bacilli. However, such organisms can probably be easily differentiated from *M. tuberculosis* by their failure to grow on medium containing penicillin.

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THE EFFECT OF INSPISSATION AND STORAGE ON ISONIAZID CONCENTRATIONS IN CULTURE MEDIA

Evidence of deterioration of isoniazid under certain conditions[1-7] has prompted some concern about the interpretation of results of *in vitro* tests of bacterial susceptibility in the use of this drug.[4, 8] The present work was undertaken with the practical objective of determining whether inspissation and storage of solutions and culture media commonly employed for this purpose affect isoniazid concentrations significantly. The amount of the biologically active form has been followed under different conditions, using a chemical method which distinguishes free from total amounts of isoniazid. Aqueous solutions as well as liquid and solid culture media containing isoniazid were analyzed before and after inspissation at 90°C. and at various intervals of storage at 37° and at 2° to 6°C.

Medium 1: Glass distilled water and 0.01 M phosphate buffers of pH 5.6 and pH 6.8 containing 20 or 1,000 γ of isoniazid per ml. were analyzed at intervals during nine weeks' storage at 2° to 6°C. Solutions in phosphate buffer of pH 6.8 (20 and 100 γ per ml.) were also analyzed before and after inspissation at 90°C. in a Levin-Brandon inspissator.

Medium 2: Sterile Proskauer and Beck type of medium containing 20 γ of isoniazid per ml. was studied at 37°C. with and without either 0.02 per cent Tween® 80 and 0.25 per cent bovine serum albumin, fraction V, or 10 per cent rabbit serum.

Medium 3: Sterile Dubos medium[9] with 0.02 per cent Tween 80 and 0.25 per cent bovine albumin, containing casein hydrolysate (N-Z amine) and glucose instead of the asparagine and glycerol of Proskauer and Beck medium, was also studied after intervals of incubation at 37°C. In addition, Dubos medium was analyzed, together with appropriate controls, after inoculation with *Mycobacterium tuberculosis*, strain H37Rv susceptible to 0.05 γ of isoniazid per ml. Bacilli were removed by filtration through sintered glass immediately after inoculation and at intervals after incubation at 37°C., and filtrates were used for analyses.

Media 4: Löwenstein-Jensen and American Trudeau Society media were analyzed before and after inspissation and at intervals after storage at 2° to 6°C. as well as at 37°C. Drug concentrations were 20 or 25 γ per ml. and 100 γ per ml. The liquid media were tubed in measured 6 ml. or 8 ml. amounts in 20 by 125 mm. screw-capped test tubes and were analyzed twice in the liquid state before inspissation: (*a*) as soon as possible after adding the drug and (*b*) after the expiration of the time required for inspissation and cooling of the remainder of the medium, i.e., at the time of the "0 hour" analyses of inspissated media.

After inspissation, extracts of coagulated media were made by taking advantage of the high water solubility of isoniazid. Weighed amounts of solidified egg medium were mashed to a paste with mortar and pestle. Small amounts of water were added at intervals and the fluid paste was painstakingly washed quantitatively into volumetric

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[7] Winder, F.: Am. Rev. Tuberc., 1956, *73*, 779.
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[9] Dubos, R., and Davis, B. D.: J. Exper. Med., 1946, *83*, 409.

TABLE 1

STABILITY OF ISONIAZID IN CULTURE MEDIUM

Figures = Per Cent Recovered

Storage Temperature and Medium	0.25 to 1.0*		4.5*		4.5*		24*		48*		72*		7 Days		15 Days		21 Days		28 Days	
	Free	Total	Free	Total	Free	Total	Free	Total	Free	Total	Free	Total	Free	Total	Free	Total	Free	Total	Free	Total
37°C. Proskauer and Beck	97	93					73	73	55	58	42	42	27	28	4	5				
37°C. Proskauer and Beck serum	102	105					92	98	71	80	61	67	33	33						
37°C. Dubos casein hydrolysate	91	96					82	88	77	83			(8 days) 38	44	21	23	14	18		
37°C. Egg	(Not inspissated)				Inpissated								(7 days)		(14 days)					
	I†		II†																	
	92	97	87	96	82	90	69	81	68	85			54	69	49	62	39	54	33	41
2-6°C. Egg							72	84	73	89			63	80	64	82	57	78	57	75

* Hours after adding drug

† I = Liquid egg medium—1 hour after adding drug

II = Liquid egg medium—4.5 hours after adding drug, i.e., at time of the first analysis of inspissated egg medium

flasks and made up to volume with water, the final dilution being 1:4 or 1:5. After standing at room temperature for thirty minutes or more with frequent mixing, fine particles of solid material were removed by centrifuging, and supernatant fluids were used for analyses. Per cent recovery was based on the original concentration of drug calculated according to the relation between the volume of liquid medium measured into tared tubes and its weight after inspissation. The effectiveness of the extraction procedure was demonstrated by triplicate extractions of the same "batch" of medium by each of two persons. Recovery was 97 to 100 per cent.

Analyses were made in duplicate by People's spectrophotometric method,[10, 4] using liquid solutions after their precipitation with zinc sulfate and sodium hydroxide. For determining total isoniazid, zinc hydroxide precipitation was preceded by hydrolysis with mild sulfuric acid. Suitable reference blanks were made from zinc hydroxide-precipitated medium without drug. At least two sets of experiments were performed with each set of conditions.

Results with distilled water and buffer solutions: No evidence of deterioration was obtained over a period of nine weeks' storage in the refrigerator. The effect of inspissation of phosphate buffer solutions appeared to depend upon the initial concentration. If 100 γ per ml. were present, recovery appeared not to be affected by inspissation, but with

[10] PEOPLES, S. A., AND ROSENFELD, S.: Fed. Proc., 1955, *14*, 377.

solutions of 25 γ per ml. only about 80 per cent could be demonstrated after inspissation.

Results with the various types of culture medium: Results with the various types of culture medium are presented in table 1 and figure 1. In Proskauer and Beck type of medium, isoniazid disappeared rapidly at 37°C. No difference was detected between amounts of free and total isoniazid, indicating destruction rather than combination. It made no difference whether or not Tween 80 and bovine serum albumin were present. Ten per cent rabbit serum, however, afforded isoniazid marked protection for the first twenty-four hours and slight protection during the second twenty-four-hour period; at the end of seven days, however, free and total isoniazid concentrations were only about 5 per cent higher than they were without serum.

Somewhat higher recoveries of both free and total isoniazid were obtained from Dubos casein hydrolysate medium than from the serum-asparagine medium except at the end of the first twenty-four hours. Inoculation with tubercle bacilli did not change concentrations of isoniazid detectable by the method of analysis used.

With the egg medium, recoveries of free and total isoniazid from the two types were similar for the first week. During the next three weeks, however, concentrations of free isoniazid in American Trudeau Society medium were rather consistently higher than in Löwenstein-Jensen medium. In both types of medium, there were suggestions that the lower concentration (20 or 25 γ per ml.) changed somewhat more rapidly than the higher (100 γ per ml.). Differences between the same concentration in the two types of medium and between the two different concentrations in the same medium, however, were not marked and, in the interest of brevity, results with the two media and the two drug concentrations are here pooled and averaged. Changes at the higher temperature, however, were decidedly greater than those which occurred upon storage in the refrigerator, and these results are tabulated and illustrated separately.

In the liquid egg medium analyzed one hour after adding the drug, recovery of total isoniazid was close to 100 per cent and that of free isoniazid, 92 per cent. Three to three and a half hours later (the period required for inspissation and cooling the remainder of the medium), the total isoniazid recovered was still close to 100 per cent, but an additional 5 per cent of the free compound had been lost. Analyses of extracts of medium coagulated by inspissation which were made immediately upon cooling indicated a further 5 per cent loss in both free and total isoniazid. Thus, the concentration of free isoniazid immediately after inspissation was 18 per cent less than the amount added.

During the next twenty-four to forty-eight hours at 37°C., a further drop of 14 per cent free and 5 per cent total isoniazid occurred and, after one week, only 54 per cent was detectable as free and 69 per cent as total isoniazid. The loss during the ensuing three weeks was progressive until, at the end of twenty-eight days, 33 per cent was recovered as the free compound (41 per cent total).

At refrigerator temperatures, for the first forty-eight hours concentrations of free and total isoniazid were only slightly higher than at 37°C. After seven days, however, recoveries of both free and total drug were about 10 per cent higher than at 37°C. During ensuing weeks, progressively greater differences between refrigerated and incubated medium occurred until, at the end of twenty-eight days, free isoniazid was 25 per cent higher, and total 34 per cent higher, in the refrigerated medium. In inspissated egg medium with an initial concentration of 25 γ or more of isoniazid per ml., about 43 per cent loss of free isoniazid can be expected during the course of four weeks' storage in the refrigerator; the greater part of this loss (37 per cent) occurs during the first seven days (18 per cent by the end of the period of inspissation and cooling).

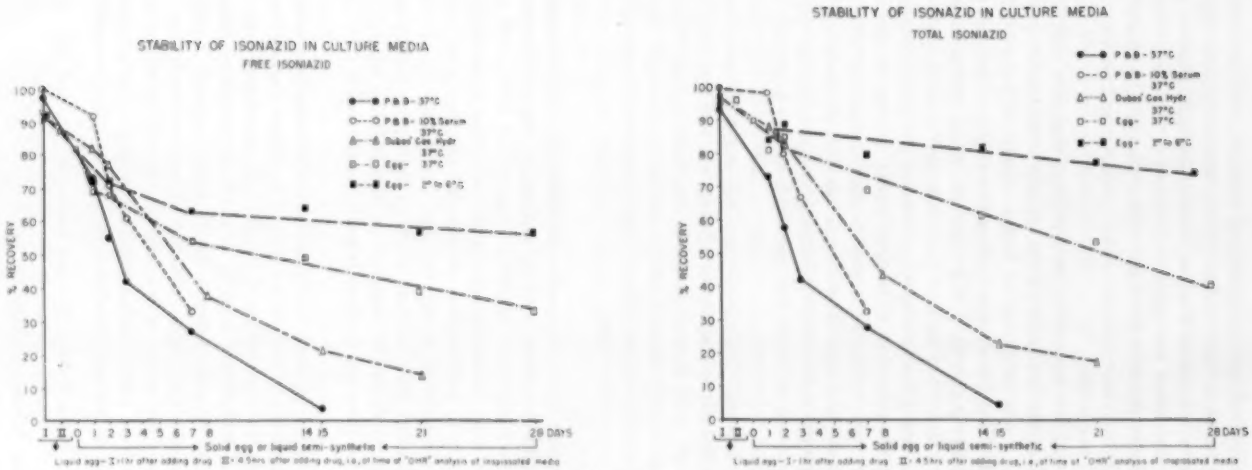

FIG. 1. Stability of isoniazid in culture media

To determine whether refrigerator storage of isoniazid medium seriously affects results of susceptibility tests of tubercle bacilli, 4 strains of varying degrees of drug resistance were planted on American Trudeau Society-isoniazid medium prepared within the previous twenty-four hours and again on portions of the same "batch" of medium which had been in the refrigerator for three weeks. Similarly, 6 strains were tested on liquid Dubos-casein hydrolysate medium freshly prepared and medium which had been in the refrigerator for two weeks.

On the solid medium, deterioration of isoniazid was suggested with 3 of the 4 strains only by more abundant multiplication on the highest concentration permitting growth, i.e., 4-plus instead of 2-plus or 3-plus, or 2-plus instead of 1-plus). The fourth strain was the susceptible H37Rv; the drug concentrations employed jumped from 0.02 γ per ml. which permitted 4-plus growth, to 0.2 γ per ml. which prevented growth. Tests of this strain on other "batches" of medium have indicated that 0.1 γ per ml. is sufficient to inhibit growth. Assuming that about 60 per cent of the initial concentration of 0.2 γ per ml., i.e., 0.12 γ, remained free after three weeks' storage of the medium, growth would not, therefore, have occurred. However, concentrations of free isoniazid in the 0.2 γ per ml. tubes in the two tests differed even less than the preceding figures indicate, for in the medium prepared twenty-four hours previous to use in the first test, even though it was refrigerated, only about 72 per cent, or 0.14 γ per ml., was free at the time of inoculation.

Deterioration in refrigerated Dubos' medium was somewhat more evident. With one of the 6 strains, the limiting concentration of drug was five times as high on the aged medium as on the fresh; with another strain, it was twice as high. With two strains, as on American Trudeau Society medium, deterioration was detected only by the greater abundance of multiplication on the highest drug concentration permitting growth; and with the remaining two strains tested, results on freshly prepared and stored medium were the same.

It is therefore evident from the results presented that: (*1*) concentrations of biologically active isoniazid in medium used for testing the susceptibility of tubercle bacilli are lower than the amounts added would indicate; (*2*) at 37°C., free isoniazid is more stable on prolonged storage in inspissated egg medium than in the liquid semi-synthetic medium; (*3*) due partly to deterioration of isoniazid and partly to combination of free drug with medium constituents during the first twenty-four hours after its addition, losses during a subsequent three or four weeks of storage at refrigerator temperatures are of minor importance in results obtained in susceptibility testing; (*4*) loss of both free and total isoniazid from egg medium is more pronounced at 37°C. than it is in the refrigerator.

The last point raises the question of why growth of cultures incubated for two to four weeks on isoniazid medium should be prevented. A partial explanation for this occurrence may be found in the experiments of Barclay and associates[11] with labeled isoniazid and in Knox's work[12] with hemin reversal of isoniazid inhibition. Barclay and his coworkers found that susceptible tubercle bacilli bind isoniazid rapidly. The amount bound reaches an initial peak at approximately sixteen to twenty-four hours. Similarly, in Knox's experiments, the action of isoniazid on tubercle bacilli could be reversed by hemin only if hemin were added within twenty-four hours. He interpreted this finding as indicating either that isoniazid reaches a site on the tubercle bacillus at which it is

[11] Barclay, W. R., Koch-Weser, D., and Ebert, R. H.: Am. Rev. Tuberc., 1954, *70*, 784.

[12] Knox, R.: J. Gen. Microbiol., 1955, *12*, 191.

inaccessible to oxidative destruction by hemin or that it quickly produces damage to the organism which is not reversible by hemin. In the present work, failure to detect greater loss of isoniazid from filtrates of inoculated than from sterile medium was probably due to the relative insensitivity of the chemical method used; that is, the amount of drug adsorbed by and removed with the susceptible organisms was so small as to be undetectable.

In the liquid media tested, combination of free isoniazid with medium constituents accounted for very little, if any, of the loss. Following the twenty-four-hour period in inspissated egg medium, however, the combination accounted for about 12 to 18 per cent of the original concentration at each period of testing.

Suggestions of slower deterioration in non-asparagine media (American Trudeau Society and Dubos casein hydrolysate) than in those containing asparagine (Löwenstein and Proskauer and Beck) are in accord with conclusions of Barry and associates[3] that asparagine contributes to the breakdown of isoniazid in media.

Evidence has been presented that deterioration of isoniazid in inspissated egg medium stored in the refrigerator for as long as three weeks before inoculation has only a minor effect on results of susceptibility tests. The reason for this circumstance appears to be that about two-thirds of the breakdown of drug occurs during the first twenty-four hours after its addition to the medium. The loss of free isoniazid during inspissation and storage is considerable. If a strain, for example, is reported as being resistant to 10 γ per ml., it has grown on medium containing actually less than 6 γ per ml., if medium on which it was tested had been stored for three or four weeks prior to inoculation (about 7 γ per ml. if the test was made within twenty-four to forty-eight hours after preparation of medium). Thus, the resistance of tubercle bacilli to isoniazid is probably considerably less than is indicated in results of susceptibility tests.

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EXPERIMENTS ON THE MECHANISM OF ACTION OF TRITON A-20 AND 1,4-DIMETHYL-7-ISOPROPYL-BICYCLO-DECAPENTANE

Therapeutic Activity on Experimental Tuberculosis of Normal and Immunized Guinea Pigs and on Experimental Murine Leprosy

Triton A-20[1-3] and 1,4-dimethyl-7-isopropyl-bicyclo-decapentane (BD.I)[4, 5] have been reported to have therapeutic activity against experimental tuberculosis without exhibiting any—or exhibiting very low—antituberculous activity *in vitro*. However, there is no clear-cut experimental evidence that the above substances act exclusively on the host. The action of triton A-20 and BD.I was studied on fixed and mobile cells of the reticuloendothelial system. Also studied was the activity of these compounds when used alone or combined with dihydrostreptomycin on experimental tuberculosis of normal and BCG-immunized guinea pigs and on experimental murine leprosy.

Trypan blue (Menkin) and india ink (Jancsó) tests: Menkin's[6] trypan blue test was used to demonstrate the action of the tested substances on the capillary permeability, and Jancsó's[7] india ink test served for the detection of acquired phagocytic activity of the capillary endothelium. Experiments were performed as previously described.[8]

A 2 per cent solution was prepared of triton A-20 and histamine, dissolved in saline solution, and of BD.I in ethyl alcohol. Each substance was tested on 10 rats (Sprague-Dawley, weighing 100 gm.). Substances were rubbed for three minutes on the previously depilated abdominal skin, and immediately thereafter the trypan blue or india ink was injected intravenously. One hour later, the intensity of increased capillary permeability and induced endothelial phagocytosis was recorded as compared with the similar phenomenon induced by intradermal injection of 10 γ of histamine. Similarly, the substances were tested on histamine-depleted animals. The animals were depleted with 48/80 as described by Brockhurst and co-workers.[9] An intense endothelial phagocytosis and increased capillary permeability were induced by histamine (++++) and BD.I (++++) on the skins of normal and depleted rats, while weak reactions (±, +) were induced with triton A-20. Similar results were obtained if 20 γ of triton A-20 were injected intradermally.

The present writers previously reported[8] that BD.I had a systemic effect on the reticuloendothelial system of rats as measured by the rate of disappearance of india ink from the blood stream. Using the same method,[8] experiments were repeated on triton A-20-treated rats.

Fifty rats were injected subcutaneously once daily with 10 mg. of triton for eight consecutive days. The last injection of triton was given thirty minutes before the india ink was injected intravenously. The rapidity of disappearance of india ink particles from the blood stream of triton A-20-treated animals was the same as for control animals.

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[6] Menkin, V.: Dynamics of Inflammation, The Macmillan Company, New York, 1950.
[7] Jancsó, M.: Orvosok Lapja, 1947, *28*, 1025.
[8] Kátó, L., and Gözsy, B.: Am. J. Physiol., 1956, *184*, 296.
[9] Brockhurst, W. E., *et al.*: J. Physiol., 1955, *129*, 205.
[10] Suter, E.: J. Exper. Med., 1952, *96*, 137.

TABLE 1

THERAPEUTIC ACTIVITY OF TRITON A-20 ALONE AND IN COMBINATION WITH DIHYDROSTREPTOMYCIN IN EXPERIMENTAL TUBERCULOSIS OF NORMAL AND IMMUNIZED GUINEA PIGS

Dihydrostreptomycin	Triton A-20	Number of Animals	Number Dead	Index of Tuberculosis
		Nonimmunized Guinea Pigs		
mg.	*mg.*			
—	—	6	3	83.5
2.5	—	6	1	76.0
—	5	6	2	72.8
2.5	5	6	1	68.2
—	25	6	1	41.5
2.5	25	6	2	24.5
—	50	6	2	43.3
2.5	50	6	1	19.5
—	100	6	6*	—
2.5	100	6	6*	—
		BCG-Immunized Guinea Pigs		
—	—	6	0	53.1
—	50	6	1	46.4
2.5	50	6	0	43.0

* All animals died within six days after treatment started.

Treatment began twenty-one days after subcutaneous infection with 0.1 mg. (wet weight) H37Rv and lasted for eight weeks. Surviving animals were killed two weeks after treatment ended. Dihydrostreptomycin and triton A-20 were given daily subcutaneously.

Phagocytic capacity of monocytes as influenced by triton A-20 in vitro and in vivo: Monocytes were obtained as described by Suter,[10] and experiments were performed as in previous studies on BD.I.[11, 12] Doses of 10 to 100 γ of triton A-20 per ml. did not increase the number of BCG organisms ingested by the monocytes *in vitro*. Monocytes of animals treated as above with triton A-20 showed no increased phagocytic activity and sera of triton A-20 animals did not affect the phagocytosis of BCG by monocytes of normal animals. Monocytes of the triton-treated animals or monocytes in the presence of triton *in vitro* did not differ morphologically from those of normal animals as seen through the contrast phase microscope. It was surprising to note that peritoneal exudate of the triton-treated animals contained markedly less numbers of monocytes as compared with the number of monocytes induced by glycogen in the peritoneal cavity of control animals. These observations confirm those of Mackaness[13] despite the fact that Mackaness used paraffin oil to induce cellular exudate.

Antituberculous activity of triton A-20 alone and in combination with dihydrostreptomycin in normal and BCG-immunized guinea pigs:

Test animals were injected intracutaneously with 10 mg. of BCG vaccine, and control animals were injected with the diluent (Sauton 1:4). Three months later, immunized and control animals were injected subcutaneously with 0.1 mg. of human tubercle

[11] GÖZSY, B., AND KÁTÓ, L.: Canad. J. Biochem. Physiol., 1956, *34*, 571.

[12] KÁTÓ, L., AND GÖZSY, B.: Canad. J. Biochem. Physiol., 1956, *34*, 580.

[13] MACKANESS, G. B.: Am. Rev. Tuberc., 1954, *69*, 690.

TABLE 2

SOME PHARMACOLOGIC AND THERAPEUTIC PROPERTIES OF TRITON A-20 COMPARED WITH 1,4-DIMETHYL-7-ISOPROPYL-BICYCLO-DECAPENTANE

	Triton A-20	1,4-Dimethyl-7-Isopropyl-Bicyclo-Decapentane
Induced endothelial phagocytosis	Weak	Intense[9, 10]
Induced capillary permeability	Weak	Intense[9]
Systemic action on reticuloendothelial system	None	Yes[9]
Phagocytosis of BCG by monocytes	Not influenced	Increased[11, 12]
Action of serum of treated animals on phagocytosis	None	Increased[11]
Multiplication of tubercle bacilli in monocytes of treated animals	Slow growth[13]	Experiments in progress
Antituberculous activity in mice when used alone	Yes[1, 2]	None[18]
Antituberculous activity in mice when combined with dihydrostreptomycin[3]	Synergistic[2]	Not synergistic[18]
Antituberculous activity in guinea pigs when used alone	Yes[3]	Yes[4, 5]
Antituberculous activity in guinea pigs when combined with dihydrostreptomycin	Synergistic	Synergistic[5, 17]
Antituberculous activity on immunized (BCG) guinea pigs	None	None (in press)[18]
Influence of antihistamine on the antituberculous activity	Not tested	Inhibition[4]
Influence on tuberculin hypersensitivity (guinea pig)	Depression[16]	Depression[18]
Activity on murine leprosy when used alone	None	None
Activity on murine leprosy when combined with Promin	None	None

bacilli (H37Rv). Treatment was started twenty-one days after infection and lasted for eight weeks. Triton and dihydrostreptomycin were administered by subcutaneous route, and control animals received the solvents. All surviving animals were killed fourteen days after treatment was ended.

At autopsy, the extent of gross tuberculous involvement was evaluated by the method of Karlson and Feldman.[14] The results are summarized in table 1. Triton A-20 showed significant antituberculous activity when used alone. This observation confirms the findings of Rees.[3] When used together with dihydrostreptomycin, a synergistic effect was observed. Triton A-20 did not enhance the effect of immunization with BCG.

[14] KARLSON, A. G., AND FELDMAN, W.: Ann. New York Acad. Sc., 1949, *52*, 637.

[15] GÖZSY, B., AND KÁTÓ, L.: Canad. J. Microbiol., 1955, *1*, 455.

[16] HART, P. D'A., *et al.*: Brit. M. J., 1952, No. 4760, 680.

[17] GÖZSY, B., AND KÁTÓ, L.: Am. Rev. Tuberc., 1956, *73*, 442.

[18] KÁTÓ, L., AND GÖZSY, B.: Christmas Meeting of Canadian Public Health Association, Toronto, 1955.

Therapeutic activity on experimental murine leprosy:

The Hawaiian strain of murine leprosy was transmitted subcutaneously to 36 Wiersing albino rats. Animals were divided into four groups. The first group received 10 mg. of triton A-20. The second received 10 mg. of a sulfone (Promin®). The third group received the same doses of triton but combined with the same amount of sulfone. The fourth group served as controls. Compounds were given daily subcutaneously. Treatment started the day of infection and lasted eight weeks, at which time the animals were killed.

At autopsy no therapeutic action was observed if triton was used alone, and triton did not increase the slight therapeutic action of sulfone. Similar experiments on 48 mice infected intraperitoneally, using the same four groupings and the same doses for six weeks of treatment, revealed no therapeutic activity by triton on the outcome of the disease. Comparative results are shown in table 2.

The experiments suggest that there are several ways to stimulate the defence processes and that this stimulation is a selective one. This selective action is certainly dependent upon physiologic characteristics of the specific cells. While the decapentane derivative (a liposoluble compound) exhibits a systemic stimulation on the reticuloendothelial system, the same action of the nonionic detergent (a water-soluble compound) on the reticuloendothelial system is more specific for the macrophages and weakly stimulatory for the capillary endothelial phagocytosis. The presence of histamine (as a mediator) is either not necessary or its activation by the test compounds is not the only way of causing stimulation. Monocytes do not contain histamine, and capillary endothelial phagocytosis can be induced even on histamine-depleted animals. Further experimental evidence is presented that the surface-active property of a compound is not a requirement for the stimulation of the cellular defenses since the same effects can be achieved with the decapentane derivative.

While it still cannot be disclaimed that triton A-20 acts on tubercle bacilli *in vivo*, its action on the host is more probable.[19] Triton A-20 showed no antituberculous activity in BCG-immunized and -challenged guinea pigs. It can be supposed that the immune principle reached a maximal level by the immunization and that the level cannot be further increased by treatment with compounds acting on the host.

It is interesting to note that, while both compounds stimulate the macrophages to an increased specific activity, neither shows therapeutic action on murine leprosy. It would appear that the cellular defenses against these two diseases are influenced by different factors and functions of the macrophages[20] and/or the environment (extracellular factors). The present experiments have given further evidence that the outcome of experimental tuberculosis can be influenced more favorably by simultaneous action on the host and the parasite than on the parasite alone.

INSTITUTE OF MICROBIOLOGY AND HYGIENE
UNIVERSITY OF MONTREAL
MONTREAL, CANADA

LÁSZLÓ KÁTÓ
BÉLA GÖZSY

September 25, 1956

[19] Experimental Tuberculosis. A Ciba Foundation Symposium. J. & A. Churchill, Ltd., London, 1955, pp. 299-310.

[20] KÁTÓ, L., AND GÖZSY, B.: Internat. J. Leprosy, 1956 (in press).

EFFECT OF GLUTAMIC ACID DERIVATIVES ON GROWTH AND INHIBITION OF MYCOBACTERIA[1,2]

It has been reported recently (GINSBURG, B., *et al.*: Arch. Biochem. Biophys., 1956, *60*, 164) that ten representative strains of defatted mycobacteria, grown on a chemically defined medium, had strikingly similar amino acid composition. It was found, however, that the glutamic acid content of the human virulent strain (H37Rv) exceeded that of the human avirulent (H37Ra) by more than 50 per cent and that of the average value for nine strains by nearly 70 per cent. It was shown, further, that glutamic acid was the most abundant amino acid in all strains.

It was deemed of interest, therefore, to undertake the studies on the metabolism of glutamic acid in mycobacteria reported in this paper.

The medium employed contained L-glutamic acid, 0.5 per cent; potassium dihydrogen phosphate, 0.5 per cent; potassium sulfate, 0.005 per cent; glycerol, 2.0 per cent; and magnesium citrate, 0.015 per cent. The medium was adjusted with 40 per cent NaOH to pH 7.0 before adding the magnesium citrate and then to pH 7.2. The organisms used were *Mycobacterium ranae*[3] and *M. smegmatis*.[4]

The compounds investigated were dissolved in the basal medium for the inhibition studies and in the basal medium lacking L-glutamic acid for the growth studies. In all cases the medium was adjusted to pH 7.2. Test tubes (16 by 100 mm., soft glass), containing the media and test substances (3 ml. final volume), were sterilized by Seitz filtration, covered with sheets of aluminum foil, and incubated. The inocula were grown on the basal medium containing L-glutamic acid. Two large loopfuls of the surface pellicle were transferred to an eight-inch test tube, glass beads were added, and the tube was shaken for ten minutes. Twenty milliliters of the appropriate medium were added, and shaking was continued for five minutes. This suspension was diluted to 150 ml., and 0.1 ml. of the final suspension was used to inoculate the tubes. The incubation temperature was 38°C.

Growth was estimated visually after four days in inhibition experiments and daily for fourteen days in growth experiments. The growth scale employed was: 0, no growth, tube clear; 1, subsurface growth or very slight surface film; 2, thin surface pellicle; 3, medium surface pellicle; and 4, heavy surface pellicle.

It may be noted from the data in table 1 that, of the twenty-seven compounds tested, L- and DL-glutamic acids, L-asparagine, and the L-, D-, and DL- forms of glutamine were the best sources of nitrogen for *M. ranae*. It is of interest that compounds which supported growth relatively poorly included D-glutamic acid and L-aspartic acid. Since D-glutamic acid was more effective at higher concentrations it appears that a glutamic acid racemase was induced in the organism. The relative inactivity of derivatives, such as α-methyl-DL-glutamic acid, may be explained possibly by steric hindrance. The approximately equal utilization of acetyl-L-glutamic acid and acetyl-D-glutamic acid may be due to the more ready racemization of the acetyl-D-glutamic acid than of D-glutamic acid. The relatively

[1] This investigation was aided by a grant from the Committee on Medical Research of the American Trudeau Society, medical section of the National Tuberculosis Association; by a research grant A-552(C3) from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service; by a grant from Swift and Company; and by a grant from the University of California.

[2] Paper No. 113. For the preceding related paper see No. 106 (GINSBURG, B., *et al.*: Arch. Biochem. Biophys., 1956, *60*, 164).

[3] Kindly furnished by Dr. L. McClaren, Medical School, University of California, Los Angeles, California.

[4] Kindly furnished by Dr. S. Froman, Olive View Sanatorium, Olive View, California.

TABLE 1

EFFECT OF COMPOUNDS RELATED TO GLUTAMIC ACID ON GROWTH OF M. RANAE ON BASAL MEDIUM LACKING GLUTAMIC ACID*

Compound	Concentration (Molar) of Compound						
	0.100	0.075	0.050	0.040	0.030	0.015	0.005
L-Glutamic acid	3	3	3	3	3	3	3
D-Glutamic acid	6	6	14	14	>14†	>14‡	>14‡
L-Glutamine	3	3	3	3	3	3	3
D-Glutamine	5	3	3	3	—§	4	5
DL-Glutamic acid	3	3	3	3	3	3	3
DL-Glutamine	4	4	4	4	4	4	4
Carbobenzoxy-L-glutamine	5	3	3	3	3	3	6
Carbobenzoxy-D-glutamine	6	6	7	7	—	>14‡	>14‡
Carbobenzoxy-L-glutamic acid‖	>14†	>14†	>14†	>14‡	>14‡	>14‡	>14‡
Acetyl-L-glutamic acid	5	5	5	9	—	>14†	14
Acetyl-D-glutamic acid	6	6	6	9	14	14	>14#
Phthalyl-DL-glutamic acid	I**	I	11	>14†	>14#	—	—
Phthalyl-DL-glutamine	I	I	6	6	6	7	>14†
α-Methyl-DL-glutamic acid	>14†	>14†	>14‡	>14‡	>14‡	>14‡	>14‡
γ-Methyl ester of L-glutamic acid	3	3	—	5	5	9	14
γ-Methyl ester of D-glutamic acid	>14‡	>14†	>14†	>14†	14	>14#	>14‡
γ-Ethyl ester of L-glutamic acid	—	6	5	5	5	9	14
L-Pyrrolidone carboxylic acid	>14#	>14#	>14†	>14†	—	—	>14‡
DL-α-Isoglutamine	—	—	4	4	4	5	5
L-Aspartic acid	5	5	5	7	7	>14‡	>14†
L-Asparagine·H_2O	3	3	3	3	3	3	5
DL-α-Aminobutyric acid	>14‡	>14‡	>14‡	>14‡	>14‡	>14‡	>14‡
γ-Aminobutyric acid·HCL	—	—	3	3	5	4	5
α-Aminoisobutyric acid	3	3	3	3	4	4	5
α-Ethyl-DL-α-aminobutyric acid	>14‡	>14‡	>14‡	>14‡	>14‡	>14‡	>14‡
Succinamide	5	5	4	4	4	4	5
γ-N-Methyl-DL-glutamine††	—	—	—	4	4	4	9
Ammonium chloride	4	3	3	3	3	3	4

* Values are expressed as days to attain full growth (*see* text for growth scale). Unless otherwise specified, all compounds were prepared by Albert Lepp (LEPP, A.: Metabolic Relationships of Glutamic and Related Compounds, Ph.D. Thesis, University of California, Los Angeles, 1956).

† Growth scale 2.

‡ Growth scale 1.

§ — = No growth.

‖ H. M. Chemical Company product.

Growth scale 3.

** I = Insoluble.

†† Monsanto Chemical Company product.

good utilization of carbobenzoxy-L- and carbobenzoxy-D-glutamines compared with that of carbobenzoxy-L-glutamic acid may be accounted for partly on the same basis but also by the utilization of the amide nitrogen. That amide and substituted amide nitrogen is readily utilized is indicated by the data shown for glutamine, isoglutamine, succinamide, and γ-N-methyl-DL-glutamine.

As shown in table 2, most of the compounds tested were inactive or only partially inhibitory at the higher levels. On the other hand, the γ-hydrazides of L- and D-glutamic acid and the β-hydrazide of DL-aspartic acid were highly inhibitory at all levels tested. Because of the possible significance of this observation in relation to the well-known action of

TABLE 2

EFFECT OF POTENTIAL INHIBITORS ON GROWTH OF M. RANAE AND M. SMEGMATIS ON BASAL MEDIUM CONTAINING 0.5 PER CENT L-GLUTAMIC ACID*

Compound	*M. Ranae* Concentration of Compound (per cent)			*M. Smegmatis* Concentration of Compound (per cent)		
	1.0	0.1	0.01	1.0	0.1	0.01
L-Glutamic acid	4	4	4	4	4	4
D-Glutamic acid	4	4	4	4	4	4
Acetyl-L-glutamic acid†	4	4	4	4	4	4
α-Methyl-DL-glutamic acid‡	4	4	4	4	4	4
p-Toluene-sulfonyl-L-glutamic acid	4	4	4	3	4	4
L-Pyroglutamic acid	4	4	4	4	4	4
DL-Pyroglutamic acid	4	4	4	4	3	4
L-Glutamic acid γ-hydrazide§	0	0	0	0	0	0
D-Glutamic acid γ-hydrazide	0	0	0	0	0	0
L-Glutamine	4	4	4	4	4	—‖
D-Glutamine	2	4	4	4	4	4
Carbobenzoxy-L-glutamine	4	4	4	2	3	4
Carbobenzoxy-D-glutamine	4	4	4	3	4	4
p-Toluene-sulfonyl-L-glutamine	4	4	4	4	4	4
Phthalyl-DL-glutamine	4	4	4	4	4	4
γ-Aminobutyric acid	1	4	4	2	3	4
DL-Ethionine sulfone	1	2	3	1	2	3
DL-Methionine sulfoximine	1	1	4	1	2	4
DL-Methionine sulfone	1	3	4	1	3	4
DL-Isopropionine#	0	2	4	1	2	3
Sodium homocysteinate	2	2	4	2	3	4
Benzal-4-amino-4-carboxybutyro-hydrazone	I**	1	2	I	1	2
γ-Glutamyl ethylamine	3	4	4	3	4	4
γ-N-Methyl-DL-glutamine	—	2	3	—	3	4
γ-Methyl-L-glutamate	0	4	4	1	4	4
α-Methyl-DL-aspartic acid‡	2	3	4	—	4	4
α-Methyl-DL-asparagine‡	2	1	1	3	2	1
DL-Aspartic acid β-hydrazide‡	0	0	1	0	0	1
L-Aspartic acid	4	4	4	4	4	4

* Values are expressed as relative growth (*see* text for growth scale) attained in four days.

† Prepared by M. P. Stoddard.

‡ Merck and Company product.

§ In autoclaved medium the limiting completely inhibitory concentration was 0.005 per cent and in Seitz-filtered medium, 0.003 per cent.

‖ — = Not tested.

Isopropyl homologue of methionine, H. M. Chemical Company product.

** I = Insoluble.

isoniazid on the growth of tubercle bacilli, further quantitative studies are in progress on the effect of these hydrazides on virulent strains of mycobacteria and on the mechanism of their action.

In summary: It was found in tests on a series of compounds that L- and DL-glutamic acids and L- and DL-glutamines were the best sources of nitrogen for *Mycobacterium ranae*. Certain N-acylated and amide derivatives, but not alpha acyl substituted compounds, were utilized but less than the former types.

Most of the compounds tested as potential inhibitors of *M. ranae* and *M. smegmatis* in the presence of L-glutamic acid were inactive or only partially inhibitory at the higher levels tested. On the other hand, the γ-hydrazides of L- and D-glutamic acid and the β-hydrazide of DL-aspartic acid were highly inhibitory at all levels.

Further studies are in progress on the mechanism of action of these inhibitory compounds and their effect on virulent strains of mycobacteria.

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BEN GINSBURG[5]
MAX S. DUNN

October 10, 1956

[5] The writers are indebted to Miss Audree Fowler for technical assistance.

Correspondence

TICE STRAIN OF BCG

To the Editor of The American Review of Tuberculosis and Pulmonary Diseases:

We have shown in a recent series of articles published in the REVIEW[1] that the substrains of BCG used in the various vaccine-producing laboratories differ profoundly one from the other in their cultural characteristics *in vitro* and in their behavior *in vivo*. Two of the cultures studied in these comparative tests had been kindly supplied to us by Dr. Sol Roy Rosenthal of the Tice Laboratory, Institution for Tuberculosis Research, University of Illinois, in Chicago, who has used both of them for the preparation of the BCG vaccine. The comparative behavior of these two cultures (labeled here Tice and BLP) is illustrated in the appended table which shows results obtained in mice with techniques identical to those reported in our earlier publications. As you will notice, the numbers of viable units injected into the experimental animals were of the same order in the four separate experiments—two for each culture—reported here.

With Dr. Rosenthal, we agree that it is important to emphasize again that all of the BCG vaccine released from the Tice Laboratory at the present time is prepared from the BLP culture.[2] In our opinion, the results obtained in mice leave no doubt that this culture is much more active *in vivo* than is the Tice substrain which was one of several substrains formerly used by Dr. Rosenthal for producing the vaccine. It is of special interest that the lyophilized batch of vaccine (BLP) used as one of the preparations in the comparative tests reported in the table proved to be approximately as active as a fresh culture (not desiccated) of this substrain and much more active than a fresh culture of the Tice substrain.

ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH
NEW YORK, NEW YORK

RENÉ J. DUBOS
CYNTHIA H. PIERCE

January 21, 1957

[1] *a*. DUBOS, R. J., AND PIERCE, C. H.: Am. Rev. Tuberc., 1956, *74*, 655.
b. PIERCE, C. H., AND DUBOS, R. J.: Am. Rev. Tuberc., 1956, *74*, 667.
c. PIERCE, C. H., DUBOS, R. J., AND SCHAEFER, W. B.: Am. Rev. Tuberc., 1956, *74*, 683.
d. DUBOS, R. J., AND PIERCE, C. H.: Am. Rev. Tuberc., 1956, *74*, 699.

[2] Personal communication from Dr. Rosenthal.

TABLE 1

COMPARATIVE BEHAVIOR OF TICE AND BLP CULTURES OF BCG

BCG Substrain	No Viable Units Injected	Organ	Numbers* of Colonies Recovered on Albumin Agar					
			Time of Sacrifice of Animal After Inoculation					
			2 weeks	3 weeks	4 weeks	5 weeks	6 weeks	7 weeks
Tice (Fresh culture)†	32×10^4	Spleen					21	
	32×10^4	Spleen					52	
	32×10^4	Spleen					61	
	32×10^4	Lungs					0	
	32×10^4	Lungs					0	
	32×10^4	Lungs					0	
Tice (Fresh culture)	7×10^4	Spleen	49	19		1		1
	7×10^4	Spleen	52	28		6		2
	7×10^4	Spleen	55	—‡		16		5
	7×10^4	Lungs	0	2		4		0
	7×10^4	Lungs	0	2		5		1
	7×10^4	Lungs	9	6		6		3
BLP (Fresh culture)	16×10^4	Spleen	560	1,500		250	38	
	16×10^4	Spleen	750	1,900		270	150	
	16×10^4	Spleen	—	—		320	220	
	16×10^4	Spleen	—	—		570	420	
	16×10^4	Lungs	19	56		400	170	
	16×10^4	Lungs	26	77		1,200	300	
	16×10^4	Lungs	27	110		1,600	320	
	16×10^4	Lungs	55	—		2,300	—	
BLP (Lyophilized vaccine)	9×10^4	Spleen	19	27	58			
	9×10^4	Spleen	27	70	210			
	9×10^4	Spleen	45	220	210			
	9×10^4	Spleen	76	270	220			
	9×10^4	Lungs	1	0	2			
	9×10^4	Lungs	2	15	13			
	9×10^4	Lungs	2	17	17			
	9×10^4	Lungs	9	22	22			

* To be multiplied by 100 for whole organ

† In Tween®-albumin medium

‡ Lost because of contamination of plate

Books

CARDIOVASCULAR SURGERY, Proceedings of International Symposium on Cardiovascular Surgery, held at Henry Ford Hospital, Detroit, Michigan. Pp. 543, W. B. Saunders Company, Philadelphia, 1955. Price, $12.75.

This book consists of the carefully edited proceedings of a symposium on cardiovascular surgery which was held at the Henry Ford Hospital, Detroit, Michigan, in March, 1955. The sixty participants in this symposium represented all but a few of the outstanding workers in the field of cardiovascular surgery during the past decade. The international nature of this symposium is attested to by the participation of eminent investigators and surgeons from Canada, England, France, Sweden, Denmark, New Zealand, and South America. One day of the symposium was devoted to the problems of congenital heart disease, one day to acquired heart disease, and one half day was spent on the surgery of the aorta and peripheral arteries. The present volume covers the same general areas.

In the section on diagnostic techniques, one is impressed by the fine radiographic equipment used by the Swedish investigators. Selective angiocardiography, the injection of contrast material directly into the heart via a catheter, is a technique seldom used in this country. However, in the hands of the Swedish investigators the results seem satisfactory and they report that there is no increase in risk as compared with the intravenous technique.

The problems arising from communications between pulmonary and systemic circulations are considered from clinical, physiologic, and pathologic points of view. The mechanism whereby balance between the two circulations is achieved in the presence of left to right shunts is clearly presented.

The panel discussions on pulmonic stenosis, interatrial septal defects, hypothermia, and the late results of mitral commissurotomy are not quite as interesting in print as they seemed at the time of the original presentations. A few of these discussions are somewhat rambling but, if one follows them carefully, many practical points can be gleaned.

Pump-oxygenators for cardiac by-pass is mentioned only in a footnote, but controlled cross circulation is described in detail. At the time of this symposium, controlled cross circulation was the only means of accomplishing successful repairs of ventricular septal defects.

The section on mitral stenosis covers many practical points on diagnosis, treatment, and late postoperative results. For mitral insufficiency a variety of approaches are described, but many of these have since been abandoned by their advocates. In the portion devoted to aortic valvular disease, the accepted clinical methods of treating stenosis and insufficiency are discussed and various experimental techniques for by-passing the aortic valve are presented. The section on the surgical treatment of aneurism of the aorta and occlusive arterial disease also includes a panel discussion on the various types of substitutes for arterial segments.

Although many of the procedures and operations described in this book are considered obsolete at the present time, it does represent a compilation of the opinions of the foremost cardiovascular authorities in the world as of March, 1955. Anyone interested in the rapidly advancing field of cardiovascular surgery will want to read this book.

GEORGE R. HOLSWADE
New York, New York

THE LIFE OF BACTERIA. Their Growth, Metabolism and Relationships. By Kenneth V. Thimann. Pp. 775, The Macmillan Company, New York, 1955. Price, $13.50.

Intended as a text for an advanced course in bacteriology, ". . . the book has come to be an attempt not only to see bacteriology as a whole—that is, as a branch of biology—but also to see it in its perspective as a development from the past and as an active area of modern investigation." The book is well written and, in the reviewer's opinion, far excels the average text in style and content. Its four sections deal successively with: 1. The Morphology and General Physiology of Bacteria; 2. The Role of Microorganisms in the Nitrogen Cycle; 3. The Metabolism of Carbohydrates; and 4. Growth and Synthesis.

The major emphasis is on bacterial metabolism, the consideration of which occupies at least half of the text. The author has done an admirable job of assembling, digesting, and presenting this aspect of the life of bacteria and, despite the rapid advances in our knowledge of bacterial metabolism, these sections should remain valuable to the advanced student and others for many years. The inclusion of chapters on topics not usually considered in texts, such as the discussion on the soil as an ecologic environment, adds to the appeal and value of the book.

Whether the author has succeeded in his aim of seeing bacteriology as a whole is certainly a debatable matter but, since criticisms based on errors of omission are as likely to reflect the reviewer's prejudices as they are the deficiencies of the book, none will be made on this score. There are errors of commission, however, the rectification of which would improve an already excellent book. Certain topics are discussed so briefly that the uninitiated reader would be left with no impressions or even with erroneous ones.

The topics referred to are mainly descriptive and their omission would improve rather than detract from the text. A case in point is the one-paragraph description of penicillin-production methods. There are instances in which, in the reviewer's opinion, stronger conclusions were drawn than were warranted from the data then available, i.e., the discussion on the chemical composition of the cell wall or the description of the structure of the spirochaetes. Finally, there are at least the usual quota of erroneous or unclear statements of varying degrees of importance, i.e., "In *Bac. anthracis* the polyglutamate capsule also confers virulence; rough forms are avirulent . . . ."; "When whole bacteria, or bacterial extracts, or indeed almost any protein, is injected into the blood of an animal, the blood vessel walls secrete a compound which corresponds in structure to the injected compound and reacts with it specifically . . . ."; with reference to heat resistance of spores ". . . .the record goes to *Cl. botulinum*"; and so on. The statement that "Among the parasites, *M. leprae* (but not *M. tuberculosis*) also attacks hydrocarbons" may particularly annoy (or amuse?) readers of this journal, especially when the author couples it with a statement some pages further along that *M. leprae* has never been successfully cultured.

For those whose specific interests are in tuberculosis and pulmonary diseases the book has nothing to offer in terms of their specialty. However, despite the criticisms made, the book should prove very profitable to those with a medical bacteriology background who wish to expand their horizons in bacteriology as a whole.

S. C. RITTENBERG
Los Angeles, California

THE LUNG AS A MIRROR OF SYSTEMIC DISEASE. By Eli H. Rubin. Pp. 288, Charles C Thomas, Springfield, Illinois, 1956. Price, $12.50.

In this text Dr. Rubin has made use of his massive experience in internal medicine and chest diseases to discuss systemic diseases with pulmonary manifestations. Illustrative cases seem to have been drawn from his own services and thereby distinctly benefit from both a convincing and entertainingly anecdotal mode of presentation and excellent long-term follow-up. One could wish, however, that the pulmonary problems had been more emphasized and general expositions more briefly treated in his discussions of the many diseases which make up the body of the text. The tendency to pay equal attention to all of the biochemical and anatomical features, while testifying eloquently to his excellence as a general internist as well as a chest physician, produces discussions which, from a general point of view, are inferior to those found in the standard medical texts and, from the point of view of a special interest in chest disease, are less exhaustive than those in texts devoted exclusively to the thorax.

The introductory chapter on roentgenographic surveys is critically informative and the closing chapters on modes of diagnosis are of great merit. The typography is handsome and readable and the many roentgenographic reproductions seem to be the best I have seen in a medical text. Perhaps the most outstanding feature of the work is the extensive, critically selected, and conveniently arranged bibliography.

ROGER M. DES PREZ
New York, New York

BOOKS RECEIVED

STUDIO CLINICO-RADIOLOGICO DEGLI ESITI DELL'ASCESSO POLMONARE. By Francesco Santagati, Nardo Calvi, and Giancarlo Rivolta. Pp. 56, Istituti Psichiatrici Provinciali di Milano, Milan.

LA THERAPEUTIQUE DE LA TUBERCULOSE PULMONAIRE CHRONIQUE PAR EXCITATION DU SYMPATHIQUE. By F. Parodi. Pp. 165, Federazione Italiana Contro la Tubercolosi, Rome, 1956.

DIAGNOSTICS PNEUMOLOGIQUES. By A. P. Jarniou. Pp. 148, G. Doin & Cie., Paris, 1956. Price, 1,350 fr.

LYMPHATICS, LYMPH, AND LYMPHOID TISSUE. By Joseph Mendel Yoffey and Frederick Colin Courtice. Pp. 510, Harvard University Press (published for The Commonwealth Fund), Cambridge, Massachusetts, 1956. Price, $10.00.

DE PROGNOSE VAN DE PNEUMONECTOMIE PATIËNT. By C. D. Laros. Pp. 220, Rijksuniversiteit te Groningen, Groningen, 1956.

SCIENTIFIC COMMUNICATIONS. Research Department, Brocades-Stheeman & Pharmacia, Amsterdam, 1956.

THE SECOND NATIONAL TURKISH TUBERCULOSIS CONGRESS. Pp. 93, National Antituberculosis Society and Turkish Tuberculosis Society, Ankara, 1956.

American Trudeau Society

Medical Section of the National Tuberculosis Association

NOTICES

FORTY-SECOND ANNUAL SESSION OF THE TRUDEAU SCHOOL OF TUBERCULOSIS—1957

The Trudeau School of Tuberculosis will present its Forty-Second Annual Session commencing Monday, June 3, and concluding Friday, June 21. The concentrated three-week course will cover all aspects of pulmonary tuberculosis and also certain phases of other chronic chest diseases, including those of occupational origin.

As in the past, the clinical material for the course will be derived from the Ray Brook State Tuberculosis Hospital, the Tupper Lake Veterans Hospital, Will Rogers Memorial, and Sanatorium Gabriels. The research laboratories of the institutions in the area and the practicing tuberculosis specialists in Saranac Lake will be called upon, as formerly, to participate in the program. There will also be participation by authoritative guest lecturers from different parts of the country.

The schedule for the 1957 course is in preparation. If you wish, a copy will be mailed to you as soon as it is available.

Inasmuch as registration is limited and applications have already been received for the 1957 session, it is suggested that those who plan to attend make early application for enrollment.

The tuition is $100, payable to the Trudeau School not later than the opening date, June 3, 1957. A few scholarships are available for those who qualify.

Application form and more detailed information may be obtained by writing to:

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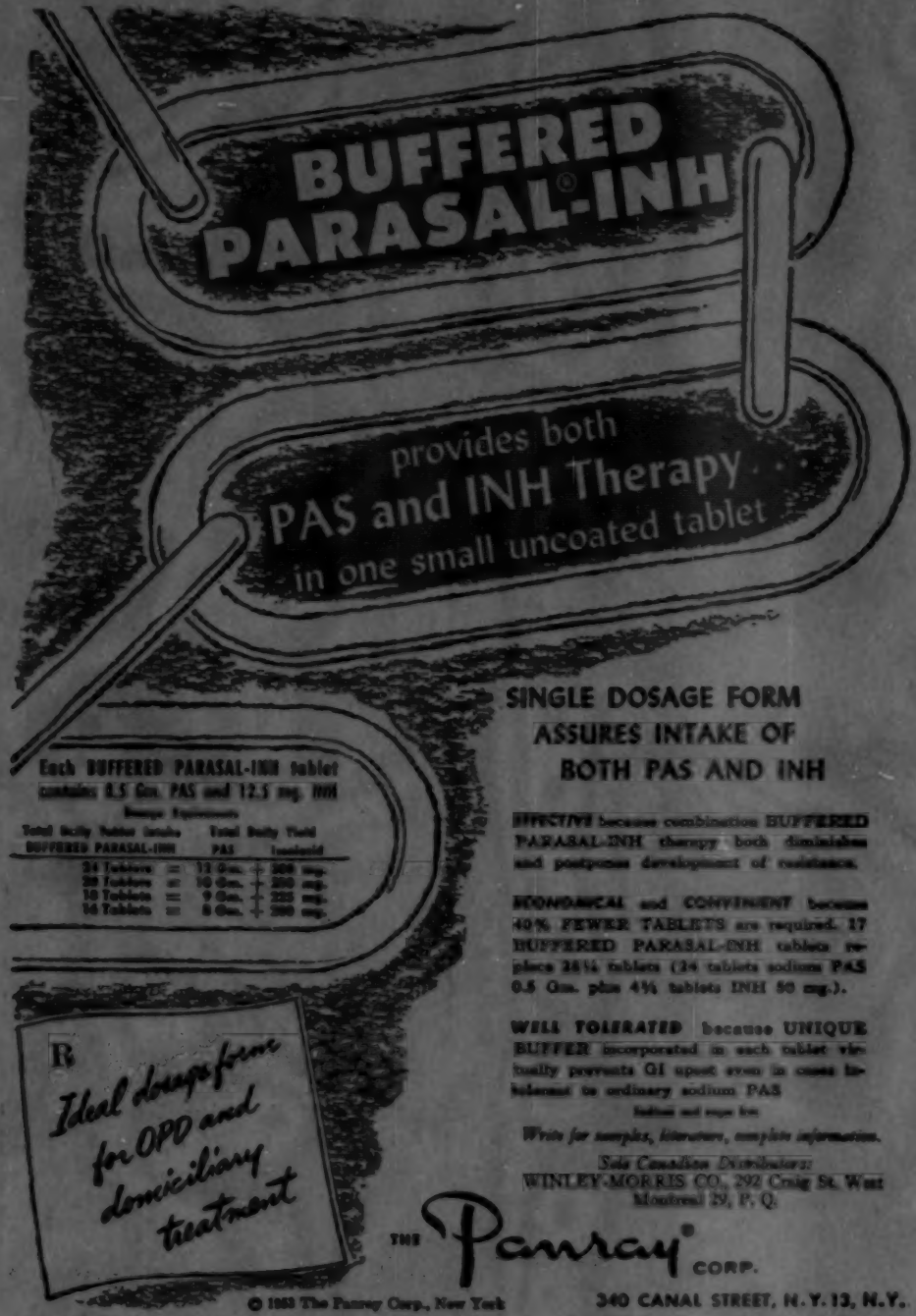
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